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PRINCIPAL INVESTIGATOR: Andrew J. Dannenberg, M.D.
Louise R. Howe, Ph.D.

CONTRACTING ORGANIZATION: Cornell University Medical College
New York, New York 10021

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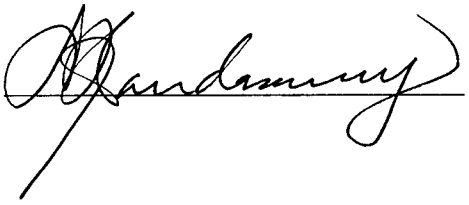

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Andrew J. Dannenberg, M.D.
Louise R. Howe, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Cornell University Medical College
New York, New York 10021

E-Mail: ajdannen@med.cornell.edu

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13. ABSTRACT ***(Maximum 200 Words)***

The inducible prostaglandin synthase cyclooxygenase-2 (Cox-2) is expressed in a variety of human cancers, but its role in breast cancer has not been definitively established. Our research was designed to test whether Cox-2 is important in the pathogenesis of mammary cancer using *Wnt-1* as a model mammary oncogene. *Wnt-1* transgenic mice exhibit mammary hyperplasia and subsequently develop mammary carcinomas. Additionally, some mouse mammary epithelial cell lines are transformed by *Wnt-1* expression. We have demonstrated upregulation of *Cox-2* gene transcription in *Wnt-1*-expressing cell lines, and in mammary tumors from *Wnt-1* transgenic mice. Our experiments suggest that Ets family transcription factors contributes to the observed *Cox-2* upregulation. Firstly, we have observed that the Ets factor PEA3 is upregulated in response to *Wnt-1* expression in C57MG mouse mammary epithelial cells, and PEA3 factors are highly expressed in tumors from *Wnt-1* transgenic mice. Secondly, we have demonstrated that PEA3 potently activates transcription of the *Cox-2* gene. In addition, we have tested the role of Cox-2 in mammary tumorigenesis by generating *Wnt-1* transgenic mice of the following *Cox-2* genotypes: (+/+), (+/-) and (-/-), and then evaluating the incidence of mammary hyperplasia and carcinoma formation in these animals. The results of these analyses are described herein.

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Table of Contents

Introduction

Cyclooxygenase (Cox) catalyzes both the synthesis of prostaglandins (PGs) and the intracellular production of mutagens from procarcinogens. The inducible form of cyclooxygenase, Cox-2, is expressed in a wide variety of human cancers and recent evidence suggests that it plays a critical role in tumorigenesis, particularly in colorectal cancer. Both epidemiological and experimental data indicate that nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit Cox activity and PG production, protect against colon cancer. In addition, experiments utilizing *Cox-2* knockout mice have shown that loss of *Cox-2* leads to a marked reduction in polyp formation in a mouse model of familial adenomatous polyposis. These results demonstrate the importance of Cox-2 in intestinal tumorigenesis. However, a role for Cox-2 in breast cancer has not been definitively established. The research to be described here had two major aims. Firstly, we undertook to test whether Cox-2 is important in the pathogenesis of mammary cancer, using *Wnt-1* as a model mammary oncogene. Transgenic mice which express *Wnt-1* from a mammary specific promoter are predisposed to develop mammary hyperplasia and subsequent carcinomas, and represent a well characterized model of mammary tumorigenesis. Female *Wnt-1* transgenic mice with the following *Cox-2* genotypes; (+/+), (+/-) and (-/-) were generated by crossing *Cox-2* (+/-) females with *Wnt-1* transgenic *Cox-2* (+/-) males. As the target mice were generated they were monitored for development of mammary hyperplasias and adenocarcinomas, to determine whether reduced *Cox-2* expression protects against formation of tumors or preneoplastic lesions. Concurrently, the molecular mechanism by which Wnt-1 upregulates *Cox-2* was investigated in cell culture models. Both the Wnt signaling pathway and expression of *COX-2* are aberrantly activated in multiple human tumors. Thus it seems likely that the mechanistic basis of Wnt-1-induced transcription of *COX-2* may be relevant to cancers of multiple organ sites.

Body

In this report, final progress will be described by reference to the tasks delineated in the Statement of Work in the initial grant proposal.

Task 1. Generate breeding stocks of *Wnt-1* transgenic and *Cox-2* knockout mice for subsequent crosses.
This was completed in year 1. All the animals were generated and genotyped by PCR analysis of tail tip DNA, then sacrificed or used in subsequent crosses as appropriate.

Task 2. Cross *Wnt-1* TG males x *Cox-2* (+/-) females to generate 10-12 *Wnt-1* TG, *Cox-2* (+/-) male F1 mice.
This was completed in year 1. 10 breeding pairs of *Wnt-1* transgenic mice and *Cox-2* (+/-) females were established, and about 100 offspring genotyped to obtain the required mice for the subsequent cross. Mice of inappropriate genotypes were sacrificed.

Task 3. Analyze *Cox-2* expression in mammary tissue from 5 *Wnt-1* TG females and 5 wild-type female litter mates.

Western blotting revealed increased levels of Cox-2 in mammary gland from *Wnt-1* transgenic mice relative to that from wildtype animals. Cox-2 protein was present at higher levels in tumor tissue compared with the hyperplastic mammary gland from *Wnt-1* transgenic mice. Figure 7 in the appended J. Biol. Chem paper shows a representative data set comparing Cox-2 protein in normal mammary gland and mammary tumor tissue from *Wnt-1* transgenic mice.

Task 4. Cross *Wnt-1* TG, *Cox-2* (+/-) males x 18 *Cox-2* (+/-) females to generate F2 *Wnt-1* TG females with the following *Cox-2* genotypes: (+/+), (+/-) and (-/-).

Using the above cross, *Wnt-1* TG, *Cox-2* (+/+) females, *Wnt-1* TG, *Cox-2* (+/-) females and *Wnt-1* TG, *Cox-2* (-/-) females were generated. All mice of inappropriate genotypes were sacrificed. We experienced problems with mortality in the *Cox-2*-null cohort, leading to failure to accumulate a substantial cohort of null mice in which to monitor tumor incidence. This was expected since it had been reported in the original publication describing generation of the *Cox-2* knockout mouse strain (1).

Task 5. Evaluate mammary hyperplasia in 5 animals each of the above F2 genotypes at 8 weeks of age.

Mammary hyperplasia was evaluated in animals of each of the following genotypes: *Wnt-1* TG, *Cox-2* (+/+), *Wnt-1* TG, *Cox-2* (+/-), and *Wnt-1* TG, *Cox-2* (-/-), using the following protocol. The 3rd and 4th pairs of mammary glands from each mouse were harvested, stained with carmine alum, and whole mounts examined microscopically. As previously described, mammary glands from *Wnt-1* transgenic mice exhibited striking epithelial hyperplasia compared with wildtype mice (2). However, we did not observe a significant difference between the mammary glands of *Wnt-1* transgenic mice with differing *Cox-2* genotypes (Figure 1). These data suggest that Cox-2 does not contribute significantly to the mammary epithelial hyperplasia observed in the *Wnt-1* transgenic mouse.

Task 6. Analyze mechanism of Cox-2 regulation by Wnt-1 in cell culture systems.

Our initial observation was that the *Cox-2* gene was transcriptionally activated in mouse mammary epithelial cell lines engineered to express *Wnt-1*. Cell lines stably expressing *Wnt-1* were generated by retroviral infection with virus encoding *Wnt-1*, and assayed for Cox-2 by Northern and Western blotting. Expresssion of *Wnt-1* resulted in elevated Cox-2 protein and RNA, due to transcriptional upregulation of the *Cox-2* gene. These data were published in Cancer Research (3)(reprint appended).

Subsequently, we focussed on identifying the molecular basis of *COX-2* upregulation in response to Wnt-1. The observation of *COX-2* upregulation in *Wnt-1*-expressing cell lines (3) and in tumor tissue resulting from *APC* mutation (4) led us to speculate that the *COX-2* gene promoter might be regulated by ß-catenin, since both *Wnt-1* expression and *APC* mutation result in ß-catenin/TCF-dependent transcriptional activation. Therefore we examined the ability of ß-catenin to activate *COX-2* promoter reporter constructs in transient transfection assays. In addition, since Ets transcription factors of the PEA3 subfamily synergise with ß-catenin to activate transcription from

promoters other than that of the *COX-2* gene (5), we were also interested to address the potential involvement of PEA3 in *COX-2* gene regulation. The results of these studies, which are briefly described here, have now been published in the Journal of Biological Chemistry (6)(reprint appended). We found that while ß-catenin only weakly activated the *COX-2* promoter, PEA3 family transcription factors were potent activators of *COX-2* transcription. Consistent with this, *PEA3* was upregulated in *Wnt-1*-expressing mouse mammary epithelial cells, and PEA3 factors were also highly expressed in tumors from *Wnt-1* transgenic mice. Thus it seems likely that Wnt-1 induces PEA3, which in turn upregulates *COX-2* transcription. In addition, promoter mapping experiments were performed to identify the region of the *COX-2* promoter required for responsiveness to PEA3. These experiments suggested that the NF-IL6 site in the *COX-2* promoter is important for mediating PEA3 responsiveness. Intriguingly, the NF-IL6 site is also important for *COX-2* transcription in some colorectal cancer lines (7), and PEA3 factors are highly expressed in colorectal cancer cell lines (5). Therefore, we speculate that PEA3 factors may contribute to the upregulation of *COX-2* expression resulting from both *APC* mutation and *Wnt-1* expression. Thus our data are relevant not only to Wnt-1-mediated induction of *COX-2*, but may also contribute to our understanding of *COX-2* misexpression in cancers of other organ sites.

Task 7. Continuously monitor *Wnt-1* TG, *Cox-2* (+/+) and *Wnt-1* TG, *Cox-2* (+/-) females for appearance of mammary tumors over a 12 month period.

17 *Wnt-1* TG, *Cox-2* (+/+) and 22 *Wnt-1* TG, *Cox-2* (+/-) female mice were monitored for tumor incidence, and maintained for up to 74 weeks. At this time, all but one of the mice had developed mammary tumors. Kaplan-Meier curves showing the survival tumor-free as a function of time in both cohorts are shown in Figure 2. There was no statistical difference between the two groups (p=0.72; statistician consulted, Dr. Howard Thaler, Memorial-Sloan Kettering Cancer Center). Thus knocking out one allele of *Cox-2* did not reduce mammary tumor incidence in *Wnt-1* transgenic mice. Due to the high mortality rate in *Cox-2*-null animals, it was not possible to adequately evaluate the consequences of knocking out both alleles of *Cox-2*. Thus, it is possible that while loss of one *Cox-2* allele was insufficient to reduce mammary tumorigenesis, loss of both alleles might confer protection, were this experiment technically feasible.

A recent report by Hla and colleagues reinforces the notion that *Cox-2* gene dosage is important (8). Liu *et al.* showed that *Cox-2* overexpression in mouse mammary gland causes tumorigenesis in breeder females, but not in virgin animals. Increased levels of *Cox-2* expression were observed in breeder female mammary tissue relative to that from virgin females, suggesting a basis for the failure to observe tumors in virgin animals, and highlighting the importance of *Cox-2* gene dosage.

It is also possible that the "negative results" obtained in this experiment may prove to be model-specific. Although funding from this grant enabled us to demonstrate that *Cox-2* was overexpressed in *Wnt-1* transgenic mammary tumors, the levels were quite modest, requiring IP westerns for detection of *Cox-2*. Future studies using other models and approaches will be required to fully establish whether inhibiting COX-2 will be useful in either the prevention or treatment of breast cancer.

Task 8. Histological analysis of mammary tumors, evaluation of *Cox-2* expression in tumors, and interpretation of results.
Both *Cox-2* expression in the tumors and interpretation of results are described above. Since no difference was observed between tumor incidence in *Cox-2* wildtype and heterozygous mice, detailed histological analysis of the tumors was not performed.

Key Research Accomplishments

- Breeding programs were established to generate numerous *Wnt-1* transgenic and *Cox-2* heterozygote mice for further breeding
- *Wnt-1* transgenic and *Cox-2* heterozygote mice were crossed to generate F1 *Wnt-1* transgenic, *Cox-2* heterozygote males for final cross
- Breeding pairs were established to generate F2 *Wnt-1* transgenic mice of genotypes *Cox-2* (+/+), (+/-) and (-/-)
- Elevated *Cox-2* expression was detected in mammary tumors from *Wnt-1* transgenic mice relative to that in wildtype mammary glands
- Morphological comparisons of mammary glands from *Wnt-1* transgenic mice of the three *Cox-2* genotypes suggested that *Cox-2* was not required for Wnt-1-induced epithelial hyperplasia
- 17 *Wnt-1*, *Cox-2* (+/+) and 22 *Wnt-1*, *Cox-2* (+/-) mice were monitored for tumor incidence for up to 74 weeks. Since tumor incidence was similar in the two cohorts, we conclude that loss of one allele of *Cox-2* is not sufficient to reduce mammary tumorigenesis in *Wnt-1* transgenic mice
- *Wnt-1* expression in mammary epithelial cell lines was shown to cause transcriptional upregulation of the *Cox-2* gene, and consequently increased prostaglandin synthesis
- We demonstrated that the Ets family transcription factor *PEA3* is upregulated in *Wnt-1*-expressing cells and tissues
- PEA3 was shown to be a potent activator of *COX-2* transcription. This observation is likely to be relevant to cancers of other organ sites, particularly colorectal cancer where both PEA3 factors and *COX-2* are upregulated

Reportable Outcomes

Manuscripts:

Howe, L.R., Subbaramaiah, K., Chung, W.J., Dannenberg, A.J. & Brown, A.M.C. (1999) Transcriptional activation of cyclooxygenase-2 in Wnt-1-transformed mouse mammary epithelial cells. *Cancer Research* **59**, 1572-1577.

Howe, L.R., Crawford, H.C., Subbaramaiah, K., Hassell, J.A., Dannenberg, A.J. & Brown, A.M.C. (2001) PEA3 is upregulated in response to Wnt-1 and activates the expression of cyclooxygenase-2. *J. Biol. Chem.* **276**, 20108-20115.

Howe, L.R., Subbaramaiah, K., Brown, A.M.C. & Dannenberg, A.J. Cyclooxygenase-2: a target for the prevention and treatment of breast cancer. (2001) *Endocrine-Related Cancer* **8**, 97-114.

Presentations:

1. Poster presented at Department of Defense Era of Hope meeting, June 2000 (abstract appended)

2. Oral presentation at Wnt meeting, 2001, New York City, May 2001 (abstract appended)

3. Invited speaker at Fourth Annual Opinion Leader Summit on "Targeted Therapies in the Treatment of Breast Cancer", Colorado, July 2001
   Presentation entitled "A Role for COX-2 Inhibitors in the Prevention and Treatment of Cancer"

4. Abstract submitted to Eicosanoids & Other Bioactive Lipids in Cancer, Inflammation and Related Diseases, Nashville, TN, October 2001 (abstract appended)

Funding applied for:

Research grant obtained from the Cancer Research Foundation of America, based on the observation of *Cox-2* upregulation in *Wnt-1*-expressing cells and tissues.
Title: Evaluation of Cox-2 as a Pharmacological Target for Breast Cancer Prevention
P.I.: Louise R. Howe, Ph.D.
(co-investigators: A.J. Dannenberg, M.D. and A.M.C. Brown, Ph.D)
Active: 1/15/00-1/14/02 (renewed for second year on re-application)

Employment or research opportunities received based on experience/training supported by this award:

Louise R. Howe, Ph.D. has been promoted to Assistant Professor of Cell Biology & Anatomy at Weill Medical College of Cornell University as of July 1, 2001.

Conclusions

In this report we describe two major avenues of investigation, both centering on the relationship between cyclooxygenase-2 (Cox-2) and breast cancer. Although the inducible prostaglandin synthase Cox-2 is known to be aberrantly expressed in multiple human cancers, its role in breast cancer remains to be definitively established. We have used *Wnt-1* as a model mammary oncogene to examine the relationship between Cox-2 and breast cancer. Our first set of experiments were designed to test whether Cox-2 is important in the pathogenesis of mammary cancer in *Wnt-1* transgenic mice. As a prerequisite for this study, we first demonstrated that *Cox-2* was upregulated in mouse mammary epithelial cells in response to *Wnt-1* expression. We extended this observation by showing that Cox-2 protein could be detected in mammary tumors from *Wnt-1* transgenic mice, but was virtually undetectable in wildtype mammary gland. Based on these observations, we generated *Wnt-1* transgenic mice of various *Cox-2* genotypes: wildtype, heterozygote and null, in order to permit comparisons of mammary hyperplasia and tumorigenesis in different *Cox-2* backgrounds. We found that mammary hyperplasia was unaltered in *Wnt-1* transgenic mice of differing *Cox-2* genotypes, suggesting that Cox-2 does not contribute to Wnt-1-induced mammary hyperplasia. In addition, we observed that knocking out one allele of *Cox-2* did not reduce mammary tumor incidence. Due to the high mortality rate, it was not possible to adequately evaluate the consequences of knocking out both alleleles of *Cox-2*. Thus it is unclear whether a total absence of Cox-2 would prevent or reduce mammary tumorigenesis. It is also important to note that although we were able to demonstrate that *Cox-2* was overexpressed in *Wnt-1* transgenic mammary tumors, the levels were quite modest, i.e., IP westerns were required to detect Cox-2. Hence, the "negative results" may prove to be model specific. Future studies using other models and approaches will be required to fully establish whether inhibiting COX-2 will be useful in either the prevention or treatment of breast cancer.

The second line of enquiry pursued was aimed at understanding the mechanistic basis of *Cox-2* upregulation by Wnt-1. Aberrant activation of the Wnt signaling pathway has recently emerged as a common event in human cancers. Initially detected in human colorectal cancers caused by mutation of the *APC* gene, activated Wnt signaling has now been detected in multiple human tumors. Thus, delineation of the mechanism by which Wnt-1 activates *Cox-2* transcription may contribute to our understanding of *COX-2* misexpression in many human cancers. *COX-2* is upregulated in response to both *APC* mutation and *Wnt-1* expression, both of which stimulate ß-catenin/TCF-dependent transcriptional activation. Therefore we speculated that the *COX-2* promoter might be responsive to ß-catenin. In addition, the effect of Ets transcription factors were tested, particularly those of the PEA3 sub-family, since the matrilysin promoter can be synergistically activated by ß-catenin and PEA3 factors (5). We found that while ß-catenin only weakly stimulated the *COX-2* promoter, PEA3 potently activated *COX-2* transcription. This observation is particularly significant since PEA3 expression is upregulated both in mammary and intestinal tumors. Thus PEA3 factors may contribute to *COX-2* misexpression in several tumor contexts.

References

1. J. E. Dinchuk *et al.*, *Nature* **378**, 406-9 (1995).
2. A. S. Tsukamoto, R. Grosschedl, R. C. Guzman, T. Parslow, H. E. Varmus, *Cell* **55**, 619-25 (1988).
3. L. R. Howe, K. Subbaramaiah, W. J. Chung, A. J. Dannenberg, A. M. C. Brown, *Cancer Research* **59**, 1572-7 (1999).
4. S. K. Boolbol *et al.*, *Cancer Research* **56**, 2556-60 (1996); S. L. Kargman *et al.*, *Cancer Research* **55**, 2556-9 (1995); C. S. Williams, M. Tsujii, J. Reese, S. K. Dey, R. N. DuBois, *Journal of Clinical Investigation* **105**, 1589-94 (2000).
5. H. C. Crawford *et al.*, *Molecular and Cellular Biology* **21**, 1370-83 (2001).
6. L. R. Howe *et al.*, *Journal of Biological Chemistry* **276**, 20108-15 (2001).
7. J. Shao, H. Sheng, H. Inoue, J. D. Morrow, R. N. DuBois, *Journal of Biological Chemistry* **275**, 33951-6 (2000).
8. C. H. Liu *et al.*, *Journal of Biological Chemistry* **276**, 18563-9 (2001).

Appendices

List of Personnel Receiving Pay from the Research Effort

Abstract presented at Department of Defense Era of Hope Meeting, June 2000

Abstract presented at Wnt Meeting 2001, New York City, May 2001

Abstract to be presented at Eicosanoids & Other Bioactive Lipids in Cancer, Inflammation and Related Diseases, Nashville, TN, October 2001

Figure 1

Figure 2

Cancer Research publication

Journal of Biological Chemistry publication

Review article published in Endocrine-Related Cancer

List of Personnel Receiving Pay from the Research Effort

Andrew J. Dannenberg, M.D.
Louise R. Howe, Ph.D.
Anthony M.C. Brown, Ph.D.
Wen Jing Chung
Jan Hart
Timothy Marmo

CYCLOOXYGENASE-2 AS A NOVEL TARGET FOR BREAST CANCER PREVENTION

Louise R. Howe, Kotha Subbaramaiah, Anthony M.C. Brown & Andrew J. Dannenberg

Strang Cancer Research Laboratory, Rockefeller University, New York, NY 10021, and Weill Medical College of Cornell University, New York, NY 10021

lrhowe@mail.med.cornell.edu

Cyclooxygenase (Cox) catalyzes both the synthesis of prostaglandins (PGs) and the intracellular production of mutagens from procarcinogens. The inducible form of cyclooxygenase, *Cox-2*, is expressed in a wide variety of human cancers and recent evidence suggests that it plays a critical role in tumorigenesis, particularly in colorectal cancer. However, a role for Cox-2 in breast cancer has not been established. Our research is designed to test whether Cox-2 is important in the pathogenesis of mammary cancer, using *Wnt-1* as a model mammary oncogene. *Wnt-1* transgenic mice exhibit mammary hyperplasia and subsequently develop mammary carcinomas. We have investigated the effect of *Wnt-1* on *Cox-2* expression in two mouse mammary epithelial cell lines, RAC311 and C57MG, which are morphologically transformed in response to *Wnt-1*. Expression of *Wnt-1* in these cell lines caused transcriptional upregulation of the *Cox-2* gene, resulting in increased levels of *Cox-2* mRNA and protein. Prostaglandin E_2 production was increased as a consequence of the elevated Cox-2 activity, and could be decreased by treatment with a selective cyclooxygenase-2 inhibitor. These experiments demonstrated that *Cox-2* is upregulated in response to *Wnt-1* expression, and thus laid the foundation for our ongoing experiments designed to test the contribution of Cox-2 to mammary tumorigenesis. We are currently generating *Wnt-1* transgenic mice of the following *Cox-2* genotypes: (+/+), (+/-), and (-/-), and will then evaluate the incidence of mammary hyperplasia and carcinoma formation in these animals. We anticipate that reduced *Cox-2* gene dosage may decrease the formation of mammary tumors.

The U.S. Army Medical Research and Materiel Command under DAMD17-98-1-8057 supported this work.

Common Involvement of PEA3 Factors in the Regulation of Wnt Target Genes

Louise R. Howe (1), Osamu Watanabe (1), Howard Crawford (2), John A. Hassell (3), Kotha Subbaramaiah (1), Andrew J. Dannenberg (1), and Anthony M.C. Brown (1)

1 Weill Medical College of Cornell University, and Strang Cancer Prevention Center, New York NY 10021, USA
2 Vanderbilt University, Nashville, TN 37232, USA
3 McMaster University, Hamilton, Ontario L8S 4K1, Canada

We are interested in identifying transcriptional targets of Wnt signaling that are relevant to tumorigenesis and in elucidating their mechanism of activation. We have previously reported upregulation of the inducible prostaglandin synthase, Cox-2, in response to Wnt1. Additionally, we have observed that the matrix metalloprotease matrilysin is induced by Wnt1 in mammary cells and tumors, consistent with previous observations in intestinal tumors from Apc^{Min} mice. We report here that the basic helix-loop-helix transcription factor Twist is also upregulated in *Wnt1*-expressing mammary cells and tumors. Our data suggest that Twist may function to suppress mammary differentiation and lactogenesis. *Twist* RNA is detected in the normal virgin mammary gland, but expression diminishes during lactogenic differentiation, showing an inverse correlation with expression of the milk protein ß-casein. In vitro, expression of *Twist* in HC11 mammary epithelial cells suppresses ß-casein induction in response to lactogenic hormones. Expression of *Wnt1* in HC11 cells induces *Twist* and similarly prevents lactogenic differentiation. In vivo such suppression of differentiation may contribute to tumorigenesis.

In investigating the mechanisms by which Wnt1 signaling regulates expression of *Cox-2*, *Matrilysin*, and *Twist*, we have found that the promoters of all three genes are regulated by ß-catenin and by ets transcription factors of the PEA3 subfamily. ß-catenin and PEA3 cause synergistic activation of the *Matrilysin* and *Twist* promoters, while PEA3 alone is a potent activator of *Cox-2* transcription. *PEA3* itself is upregulated in C57MG mouse mammary epithelial cells expressing *Wnt1*, and is highly expressed in tumors from both *Wnt1* transgenic mice and Apc^{Min} mice. Our data suggest that coordinate regulation by ß-catenin and PEA3 factors may be a common mechanism by which Wnt target genes are induced.

PEA3 is Upregulated in Response to Wnt1 and Activates COX-2 Expression

LOUISE R. HOWE[1,2], Howard C. Crawford[3], Kotha Subbaramaiah[2,4], John A. Hassell[5], Andrew J. Dannenberg[2,4] & Anthony M.C. Brown[1,2]

[1] Dept of Cell Biology and Anatomy, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021. [2] Strang Cancer Research Laboratory at the Rockefeller University, New York, NY 10021. [3] Dept of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232. [4] Dept of Medicine, Weill Medical College of Cornell University, New York, NY 10021. [5] Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario L86 4K1, Canada.

Cyclooxygenase-2 (COX-2) is aberrantly expressed in intestinal tumors resulting from *APC* mutation in both rodents and humans. Additionally, we have reported transcriptional upregulation of *Cox-2* in mouse mammary epithelial cells in response to *Wnt1* expression. Since ß-catenin stabilization is a consequence of both *APC* mutation and Wnt signaling, we speculated that the *COX-2* promoter might be transcriptionally regulated by ß-catenin. Here we show that while ß-catenin only weakly activates the *COX-2* promoter, Ets transcription factors of the PEA3 subfamily are potent activators of *COX-2* transcription. Consistent with this, *PEA3* is upregulated in *Wnt1*-expressing mouse mammary epithelial cells, and PEA3 factors are highly expressed in tumors from *Wnt1* transgenic mice, in which *Cox-2* is also upregulated. Promoter mapping experiments suggest that the NF-IL6 site in the *COX-2* promoter is important for mediating PEA3 responsiveness. The NF-IL6 site is also important for *COX-2* transcription in some colorectal cancer lines (Shao et al. (2000) J.Biol. Chem. 275: 33951-33956), and PEA3 family members are highly expressed in human colorectal cancer cell lines. Therefore, we speculate that PEA3 factors may contribute to the upregulation of *COX-2* expression resulting from both *APC* mutation and *Wnt1* expression.

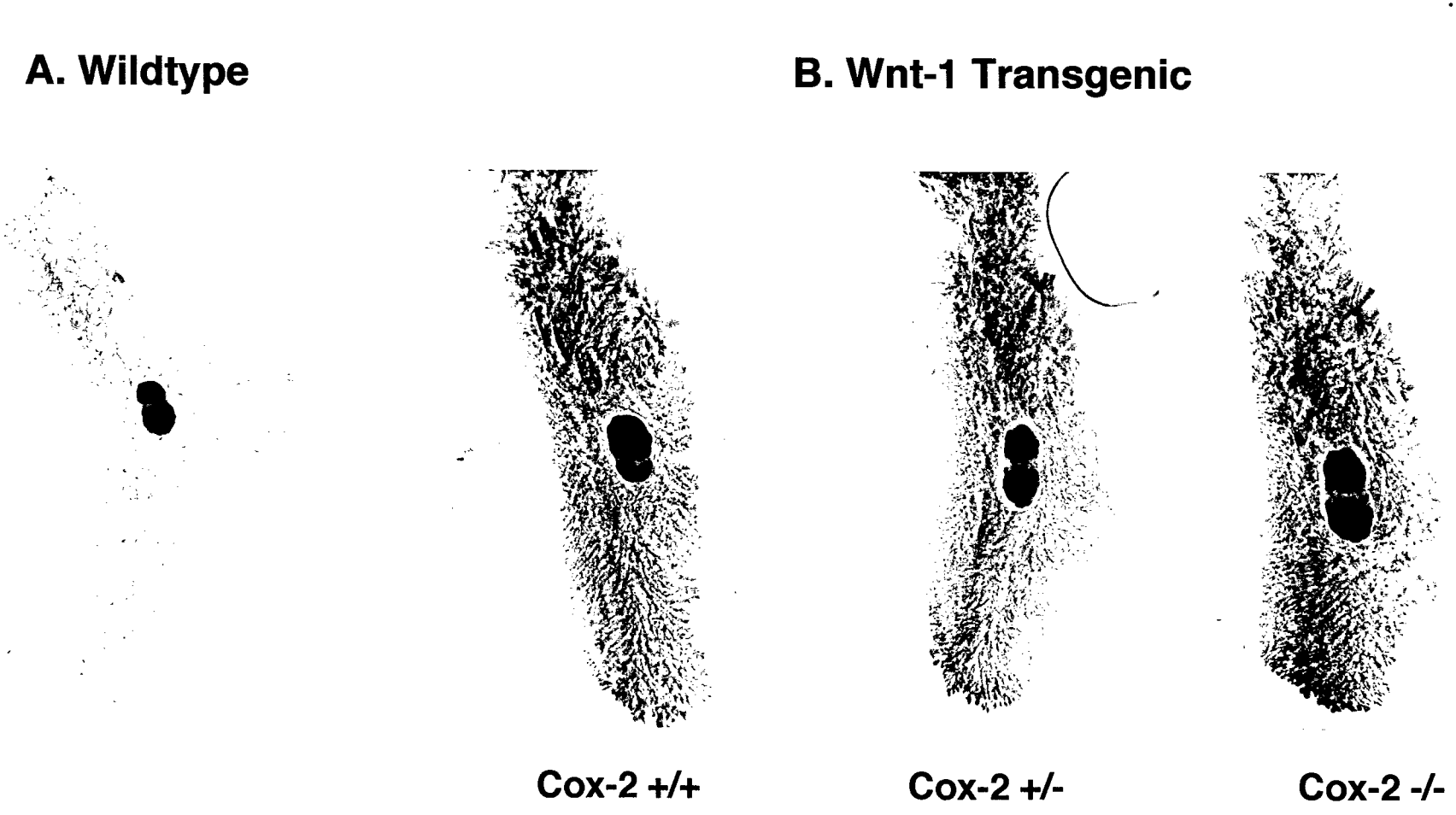

Figure 1. Whole Mount Analysis of Mammary Glands.
Epithelial hyperplasia was compared in mammary glands from mice of various genotypes, by staining the 4th inguinal mammary glands with carmine alum and examining the stained mammary glands as whole mounts. Panel A shows a wildtype mammary gland. Shown in panel B are glands from *Wnt-1* transgenic mice with varying *Cox-2* genotypes. Expression of the *Wnt-1* transgene causes marked hyperplasia (compare panels A and B), but altered *Cox-2* gene dosage does not significantly affect *Wnt-1*-induced hyperplasia.

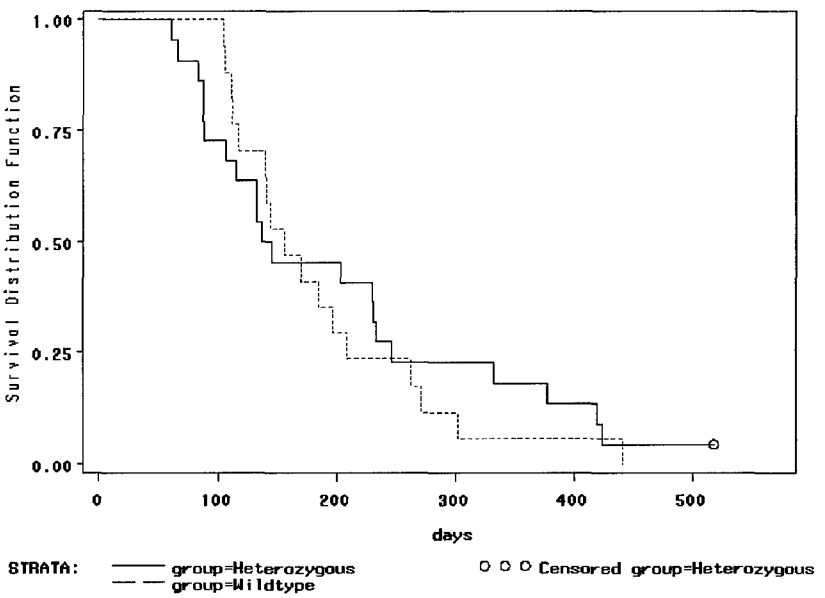

Figure 2. Tumor Incidence in Wnt-1 Transgenic Mice
Tumor incidence was measured in mice of the following genotypes; *Wnt-1* TG, *Cox-2* (+/+) and *Wnt-1* TG, *Cox-2* (+/-). Survival tumor-free was plotted as a function of mouse age (days) as Kaplan-Meier curves.There was no significant difference between tumor incidence in the two cohorts (p=0.72).

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Transcriptional Activation of *Cyclooxygenase-2* in Wnt-1-transformed Mouse Mammary Epithelial Cells[1]

Louise R. Howe, Kotha Subbaramaiah, Wen Jing Chung, Andrew J. Dannenberg, and Anthony M. C. Brown[2]

Strang Cancer Research Laboratory, The Rockefeller University, New York, New York 10021 [L. R. H., K. S., W. J. C., A. J. D., A. M. C. B.], and Departments of Cell Biology and Anatomy [L. R. H., A. M. C. B.] and Medicine [K. S., W. J. C., A. J. D.], Weill Medical College of Cornell University, New York, New York 10021

ABSTRACT

***Wnt-1* acts as a mammary oncogene when ectopically expressed in the mouse mammary gland. *APC* is a tumor suppressor gene, mutations in which cause intestinal tumorigenesis in humans and rodents. Both *Wnt-1* expression and *APC* mutation activate a common signaling pathway involving transcriptional activation mediated by β-catenin/Tcf complexes, but few targets relevant to carcinogenesis have yet been identified. Expression of the inducible prostaglandin synthase cyclooxygenase-2 appears critical for intestinal tumorigenesis resulting from *APC* mutation, suggesting that *cyclooxygenase-2* might be a transcriptional target for β-catenin/Tcf complexes. Here, we have investigated the effect of Wnt-1 on *cyclooxygenase-2* expression. *Wnt-1* expression in the mouse mammary epithelial cell lines RAC311 and C57MG induces stabilization of cytosolic β-catenin and morphological transformation. Expression of *Wnt-1* in these cells caused transcriptional up-regulation of the *cyclooxygenase-2* gene, resulting in increased levels of cyclooxygenase-2 mRNA and protein. Prostaglandin E_2 production was increased as a consequence of the elevated cyclooxygenase-2 activity and could be decreased by treatment with a selective cyclooxygenase-2 inhibitor. Cyclooxygenase-2 thus appears to be a common downstream target for *APC* mutation and *Wnt-1* expression. In view of the critical role of cyclooxygenase-2 in intestinal tumorigenesis, *cyclooxygenase-2* up-regulation in response to Wnt signaling may contribute to *Wnt*-induced mammary carcinogenesis.**

INTRODUCTION

Wnt-1 was originally identified as a mammary oncogene activated by proviral insertions of mouse mammary tumor virus (1–3). Ectopic expression of *Wnt-1* under the control of a mouse mammary tumor virus promoter leads to extensive mammary hyperplasia and subsequent generation of adenocarcinomas in mice (4). Cell culture experiments demonstrate that multiple *Wnt* gene family members including *Wnt-1* can cause partial cellular transformation of some epithelial and fibroblastic cell lines (5–12). Collectively, these data implicate *Wnt-1* as an oncogene when inappropriately expressed. Several *WNT* gene family members have been found to be overexpressed in a proportion of human breast cancers and may therefore contribute to carcinogenesis in humans (13–17).

The *Wnt-1* gene encodes a secreted protein that functions as an extracellular ligand capable of promoting mitogenesis (18–21). Wnt-1 appears to signal via a unique pathway, thought to be initiated by interaction of Wnt-1 with a member of the Frizzled family of seven-transmembrane receptors, leading to stabilization of a cytosolic pool of β-catenin (22). Accumulated β-catenin can translocate to the nucleus, interact with Tcf transcription factors, and thereby mediate transcriptional activation (11, 23–31).

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[2] To whom requests for reprints should be addressed, at Strang Cancer Research Laboratory, The Rockefeller University, Box 231, 1230 York Avenue, New York, NY 10021. Phone: (212) 734-0567, extension 232; Fax: (212) 472-9471; E-mail: amcbrown@mail.med.cornell.edu.

β-Catenin/Tcf-mediated transcriptional activation has recently been implicated in human carcinogenesis. Elevated cytosolic β-catenin and transcriptionally active β-catenin/Tcf complexes have been detected in both colon carcinomas and melanomas (32–34). β-Catenin accumulation can occur as a consequence of mutation of either the *β-catenin* gene itself or the tumor suppressor gene *APC*, because wild-type APC protein contributes to β-catenin destabilization (32–36). Mutations in *APC* cause intestinal tumorigenesis in humans and mice. Although the molecular mechanism by which *APC* mutation induces tumorigenesis is unclear, many data implicate cyclooxygenase enzymes in this process (37). Cox-1[3] and Cox-2 are constitutively expressed and inducible isoforms of prostaglandin synthase, respectively (gene symbols, *Ptgs1* and *Ptgs2*; Ref. 38). *COX-2* expression has been detected in intestinal tumors of both mice and humans with *APC* mutations (39–41). Genetic ablation of the *Cox-2* gene or pharmacological inhibition of Cox-2 activity dramatically reduces the incidence of intestinal tumors in *Apc* mutant mice (42).

Thus, both *APC* mutation and ectopic *Wnt-1* expression can cause tumorigenesis, and this may be, at least in part, via a common signaling pathway involving β-catenin/Tcf complexes. Furthermore, Cox-2 appears critical for tumor formation resulting from *APC* mutation. Consequently, we reasoned that Cox-2 might also be a target for Wnt-1 signaling and might potentially contribute to *Wnt-1*-induced mammary tumorigenesis. We therefore tested the effect of *Wnt-1* expression on Cox-2 in mouse mammary epithelial cells. Here we show that *Wnt-1* expression in RAC311 and C57MG cells causes increased transcription of *Cox-2*, resulting in elevated Cox-2 protein levels. An increase in PGE_2 synthesis is also observed in *Wnt-1*-expressing cells, which can be reversed by treatment with a selective Cox-2 inhibitor. These data may be significant not only in terms of *Wnt*-mediated carcinogenesis in the mouse but also in relation to human cancers in which components of the Wnt signaling pathway are activated.

MATERIALS AND METHODS

Cell Culture. Two mouse mammary epithelial cell lines were used, C57MG (43) and RAC311, a clonal subline derived from RAC311c (44, 45). RAC311 cells were grown in DMEM (4.5 g/l D-glucose) containing 10% fetal bovine serum (Life Technologies, Inc.), 100 units/ml penicillin, and 100 μg/ml streptomycin. C57MG growth medium was supplemented with 10 μg/ml insulin (Sigma). Cells were infected with MV7 or MVWnt-1 retrovirus using helper-free virus stocks as described previously (21). Approximately 50–100 G418-resistant colonies were pooled to generate the pooled populations designated RAC/MV7, RAC/Wnt-1, C57/MV7, and C57/Wnt-1. A clonal subline of RAC/Wnt-1 was derived by limiting dilution, selected on the basis of highly transformed morphology, and designated RAC/Wnt-1 #9. For cell lysate and RNA preparation, cells were plated at 1×10^6 cells per 10-cm dish and grown until MV7-infected control cells were confluent (5 days for RAC/MV7; 4 days for C57/MV7). DFU was a generous gift of the Merck Frosst Center for Therapeutic Research (Quebec, Canada).

Cell Lysate Preparation and Analysis. For Wnt-1 protein analysis, ECM fractions were prepared after removing the cells from the dishes by incubation

[3] The abbreviations used are: Cox, cyclooxygenase; PGE_2, prostaglandin E_2; DFU, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5*H*)-furanone; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

with Dulbecco's PBS (Life Technologies, Inc.) containing 2 mM EDTA. ECM remaining on the plates was solubilized in boiling Laemmli SDS sample buffer and stored at −20°C. For analysis of cytosolic β-catenin levels, lysates were prepared as described (46), and total protein was assayed using Bio-Rad Protein Assay reagent. For Cox-2 protein analysis, lysates were prepared essentially as described (47). Cells were washed twice with PBS and harvested in lysis buffer containing 150 mM NaCl, 100 mM Tris-Cl (pH 8.0), 1% Tween 20, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 mM diethyldithiocarbamic acid. After one cycle of freeze-thawing at −20°C, cells were sonicated (three times for 15 s each time) on ice, then debris was pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C. Supernatants were stored at −80°C, and proteins were assayed using a Lowry-based protein assay kit (Sigma).

For Western analysis, samples were subjected to SDS-PAGE as follows: Wnt-1 ECM fractions, 10% gel; β-catenin samples, 8% gel, 5 μg of protein; Cox-2 lysates, 10% gel, 50 μg of protein. Proteins were transferred to polyvinylidene fluoride membrane (Immobilon; Millipore), blotted with anti-Wnt-1 antibody (MC123; Ref. 21), anti-β-catenin antibody (Transduction Laboratories; Ref. 46), or anti-Cox-2 antibody (715; Ref. 47), and developed with Amersham enhanced chemiluminescence reagents. The anti-Cox-2 antibody 715 was a rabbit polyclonal antibody, raised against the unique 18-amino acid sequence from the COOH-terminal region of human Cox-2, which does not react with Cox-1.

RNA Preparation and Northern Blotting. RNA was prepared from confluent cells using RNAzol B (Tel-Test, Inc.) according to the manufacturer's instructions. Twenty μg of RNA were subjected to electrophoresis in 1% agarose/formaldehyde/3-[*N*-morpholino]propanesulfonic acid gels and transferred to Zeta-Probe membrane (Bio-Rad). Radiolabeled random-primed probes were prepared using the Rediprime DNA labeling system (Amersham), and hybridization was undertaken at 65°C in 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS, and 1 mM EDTA (48). Washes were performed in 40 mM Na_2HPO_4 (pH 7.2), 1% SDS at 65°C. Probes used were murine *Cox-2* (TIS-10; a gift from H. R. Herschman, University of California at Los Angeles, Los Angeles, CA), murine *Cox-1* (a gift from W. L. Smith, Michigan State University, East Lansing, MI), and murine GAPDH (obtained from A. Ashworth, Institute of Cancer Research, London, England). GAPDH was used to demonstrate equal loading of each lane.

Nuclear Run-Ons. Nuclei were prepared, and nuclear run-ons were performed as described (47).

Autoradiographic exposures of both Northern blots and nuclear run-ons were quantitated by analysis on a Macintosh computer using the public domain NIH Image program (developed at the United States NIH and available on the Internet at http://rsb.info.nih.gov/nih-image/). Values obtained were normalized to those obtained for GAPDH and 18S rRNA for Northern blots and nuclear run-ons, respectively.

PGE_2 Assays. Cells were plated in 12-well plates at 4×10^4 cells/well and grown to confluence. Culture medium was collected and assayed for PGE_2 by enzyme immunoassay (Cayman Co., Ann Arbor, MI). To assay PGE_2 production in the presence of excess arachidonic acid (AA "spiked"), cells were incubated with fresh medium containing 10 μM arachidonic acid for 30 min, and then this medium was harvested and assayed as above. For experiments assaying the effect of DFU on PGE_2 production, cells were plated in 6-cm dishes at 3×10^5 cells/dish. DFU was added in fresh medium 72 h after plating and readded at 96 h. PGE_2 production was assayed at 120 h after plating, at which time control cells were confluent.

RESULTS AND DISCUSSION

To examine the effect of Wnt-1 on *Cox-2* expression, we generated fresh cell populations expressing *Wnt-1* by infection of the mouse mammary epithelial cell lines C57MG and RAC311 with retrovirus encoding Wnt-1 (MVWnt-1) or control retrovirus (MV7). As observed previously (5, 6), both C57/Wnt-1 and RAC/Wnt-1 cells appeared morphologically transformed and grew to higher cell densities than control cells (C57/MV7 and RAC/MV7, respectively). An additional clonal subline, RAC/Wnt-1 #9, was generated from RAC/Wnt-1 by limiting dilution and selected because of its high degree of morphological transformation. Western blot analysis using an anti-Wnt-1 antibody revealed that RAC/Wnt-1 #9 produced more Wnt-1 protein than the pooled RAC/Wnt-1 population (Fig. 1*A*). Therefore, we included both RAC/Wnt-1 and RAC/Wnt-1 #9 in subsequent analyses. As demonstrated previously, expression of *Wnt-1* in C57MG led to accumulation of uncomplexed cytosolic β-catenin (Refs. 46 and 49; data not shown). In addition, cytosolic β-catenin was elevated in RAC311 cells expressing *Wnt-1* (Fig. 1*B*), and higher levels were detected in RAC/Wnt-1 #9 relative to RAC/Wnt-1, correlating with relative Wnt-1 protein production.

Cox-2 protein levels in control and *Wnt-1*-expressing cell lines were analyzed by Western blotting (Fig. 2). C57/MV7 exhibited a markedly higher basal amount of Cox-2 than RAC/MV7, in which Cox-2 protein was virtually undetectable. In both C57MG and RAC311 cell lines, however, expression of *Wnt-1* led to an increase in Cox-2 protein, and Cox-2 protein was more abundant in RAC/Wnt-1 #9 than in RAC/Wnt-1, correlating with *Wnt-1* expression levels. Analysis of Cox-2 RNA by Northern blotting demonstrated that

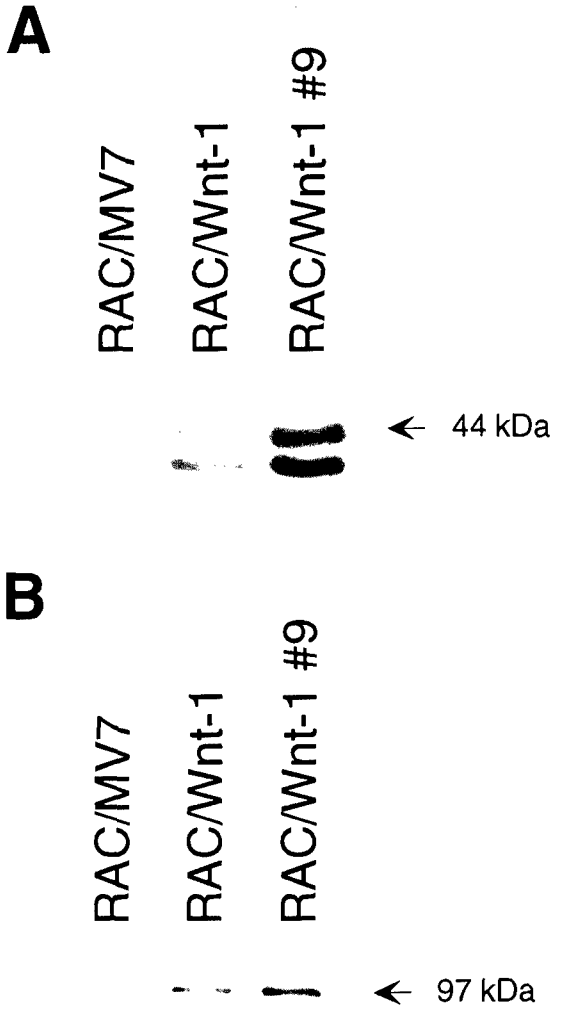

Fig. 1. Characterization of RAC311 cells expressing *Wnt-1*. RAC/MV7, RAC/Wnt-1, and RAC/Wnt-1 #9 cells were generated by retroviral infection as described in "Materials and Methods." Cells were analyzed by Western blotting for Wnt-1 protein and cytosolic β-catenin levels. *A*, *Wnt-1* expression. ECM fractions were prepared and assayed for Wnt-1 protein as described in "Materials and Methods." Anti-Wnt-1 antibody MC123 detected two bands of M_r 42,000 and M_r 44,000 in *Wnt-1*-expressing cells, as observed previously (71). These represent differentially glycosylated forms of Wnt-1 protein (20). No Wnt-1 protein was detected in ECM from control RAC/MV7 cells. *B*, cytosolic β-catenin. Cytosol fractions were prepared from cells and assayed for β-catenin as described in "Materials and Methods." The position of a M_r 97,000 molecular weight marker is shown.

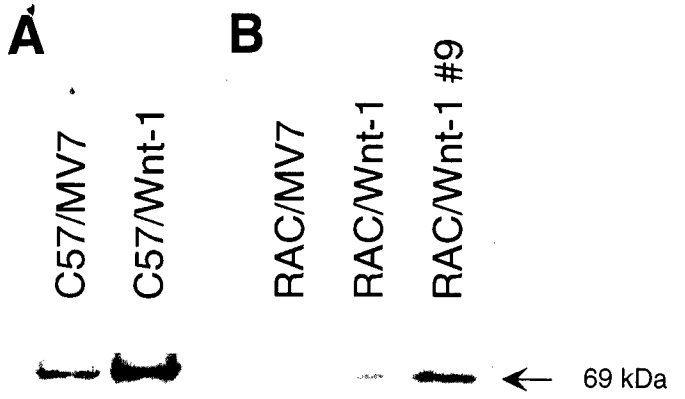

Fig. 2. Cox-2 protein is increased by *Wnt-1* expression. Lysates were prepared from C57MG-derived cells (*A*) and RAC311-derived cells (*B*). Fifty μg of lysate were analyzed by Western blotting for Cox-2 as described in "Materials and Methods," using rabbit polyclonal anti-Cox-2 antibody 715. Data shown in *A* and *B* are from separate experiments. The position of a M_r 69,000 molecular weight marker is shown.

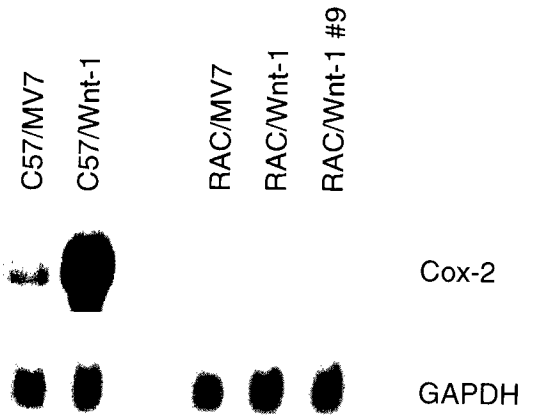

Fig. 3. Cox-2 mRNA is increased in cells expressing *Wnt-1*. Total RNA was prepared from cells, and 20 μg of each RNA sample were analyzed by Northern blotting as described in "Materials and Methods." The blot was probed sequentially with a murine *Cox-2* probe and a murine GAPDH probe. Cox-2 signals were quantitated using the program NIH Image and normalized to those obtained from GAPDH probing. Values obtained are expressed relative to the control MV7-infected cell line in each case. *C57/MV7*, 100%; *C57/Wnt-1*, 500%; *RAC/MV7*, 100%; *RAC/Wnt-1*, 174%; *RAC/Wnt-1 #9*, 327%.

Cox-2 mRNA levels closely reflected the changes observed in Cox-2 protein (Fig. 3), suggesting the effect of Wnt-1 on Cox-2 was likely to be due to transcriptional activation of the *Cox-2* gene. To test this directly, nuclear run-on assays were performed. These and subsequent assays were performed in the RAC311-derived cell lines in preference to C57MG-derived lines, because the latter tend to lose *Wnt-1* expression during continuous culture.[4] The rates of transcription from the *Cox-2* gene in RAC/Wnt-1 and RAC/Wnt-1 #9 were increased to 270 and 400%, respectively, relative to that in RAC/MV7 (Fig. 4), mirroring the differences observed in Cox-2 RNA and protein. Thus, expression of *Wnt-1* in RAC311, and most likely C57MG, causes transcriptional activation of the *Cox-2* gene.

Cox-2 is an inducible isoform of prostaglandin synthase (38). Thus, one predicted functional consequence of *Cox-2* up-regulation would be an increase in prostaglandin synthesis, of which PGE_2 is the predominant eicosanoid produced by most epithelial cells. We therefore assayed PGE_2 production in RAC311-derived cell lines. Spontaneous production of PGE_2 in RAC/Wnt-1 and RAC-Wnt/1 #9 was increased by 240 and 420%, respectively, over that in RAC/MV7 (Fig. 5, "spontaneous"). Spontaneous PGE_2 production was also measured in C57MG-derived lines and was increased ~100% in C57/Wnt-1 cells relative to C57/MV7 (data not shown). Given that arachidonic acid is the substrate from which cyclooxygenases synthesize prostaglandins, incubation of cells with excess arachidonic acid can increase PGE_2 production. In our experiments, treatment of the cells with arachidonic acid increased the absolute amounts of PGE_2 synthesis (Fig. 5, "AA spiked"), but PGE_2 production was still elevated in *Wnt-1*-expressing cells relative to RAC/MV7. This suggests that the measured spontaneous synthesis rates reflected relative levels of Cox-2 activity in the cells, rather than differential availability of arachidonic acid.

The observed increases in PGE_2 synthesis in *Wnt-1*-expressing cells could also be a consequence of changes in the level of Cox-1. Although *Cox-1* is constitutively and ubiquitously expressed, there have been reports of ligand-induced *Cox-1* up-regulation (50–53). Therefore, we addressed the involvement of Cox-1 using two assays: (*a*) we measured Cox-1 mRNA by Northern blotting and found little or no increase in Cox-1 mRNA in RAC/Wnt-1 and C57MG/Wnt-1, respectively, relative to control cells (Fig. 6); and (*b*) we tested the relative contribution of Cox-1 and Cox-2 to PGE_2 production in the RAC311-derived cell lines by using DFU, a selective Cox-2 inhibitor. DFU has at least a 1000-fold specificity for Cox-2 relative to Cox-1 in tissue culture cells (54). RAC/MV7, RAC/Wnt-1. and RAC/Wnt-1 #9 were treated with varying concentrations of DFU for 48 h, and culture supernatants were then assayed for PGE_2. A dose-dependent inhibition of PGE_2 production was observed, with 1 μM DFU being sufficient to reduce PGE_2 production to approximately the same basal level in all three cell lines (Fig. 7). Higher concentrations of DFU did not cause any additional inhibition of PGE_2 synthesis. The residual PGE_2 production observed in all cell lines after inhibition of Cox-2 with DFU is presumed to reflect Cox-1 activity. Because the amount of Cox-1-mediated PGE_2 synthesis is apparently constant in all three cell lines, we conclude that the enhanced production of PGE_2 in *Wnt-1*-transformed cells is attributable to increased Cox-2 activity, consistent with the observed differences in Cox-2 RNA and protein levels. Cell morphology was unaffected by treatment with DFU (data not shown). The failure of DFU to affect morphological transformation of *Wnt-1*-expressing cells suggests that elevated prostanoid pro-

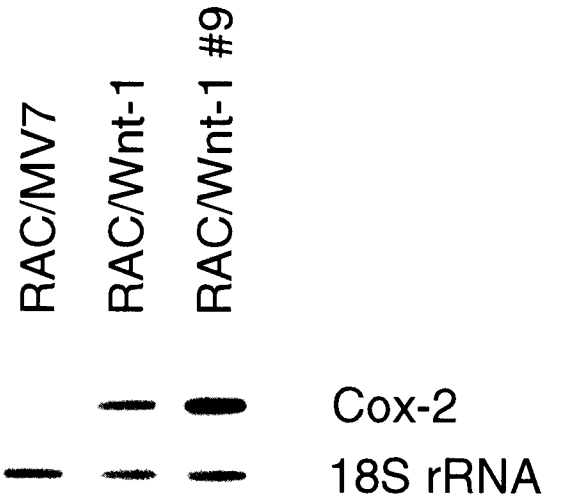

Fig. 4. *Cox-2* transcription is up-regulated in RAC311 cells expressing *Wnt-1*. Nuclei were prepared, and nuclear run-on assays were performed as described in "Materials and Methods." Labeled nascent transcripts were hybridized to 18S rRNA and *Cox-2* cDNAs, which were immobilized on nitrocellulose. Signals were quantitated using the program NIH Image, and Cox-2 was normalized to 18S rRNA. Values obtained are expressed relative to RAC/MV7. *RAC/MV7*, 100%; *RAC/Wnt-1*, 267%; *RAC/Wnt-1 #9*, 399%.

[4] A. M. C. Brown, unpublished observations.

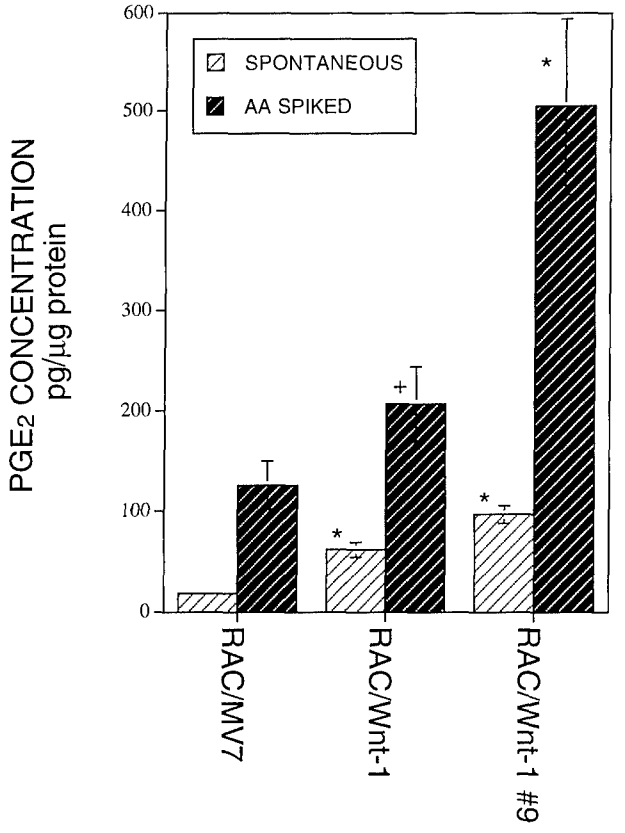

Fig. 5. PGE_2 production is increased by expression of *Wnt-1*. Cells were grown for 5 days after plating to achieve confluence, during which time no medium change was performed. Growth medium from the cells was harvested and assayed using enzyme immunoassay to measure spontaneous PGE_2 production (*SPONTANEOUS*, light hatching). Cells were incubated for an additional 30 min in fresh medium containing 10 μM sodium arachidonate. The medium was then collected and assayed for PGE_2 (*AA SPIKED*, dark hatching). Results were normalized to μg of protein obtained from the cells after harvesting of medium. Results shown are mean values of six replicates; *bars*, SD. PGE_2 production from both *Wnt-1*-expressing cell lines was significantly greater than that from RAC/MV7 control cells (*, $P < 0.001$; +, $P < 0.003$).

duction by Cox-2 is not necessary for maintenance of the transformed phenotype of these cells *in vitro*.

We have shown here that expression of *Wnt-1* in two mammary epithelial cell lines causes elevated expression and activity of Cox-2, via transcriptional activation, resulting in increased PGE_2 synthesis. The *Cox-2* gene was initially identified as an early response gene up-regulated in response to phorbol ester and serum and was subsequently found to be induced by multiple agents, particularly during inflammatory responses (38). A large body of evidence has accumulated implicating Cox-2 in intestinal carcinogenesis. *COX-2* expression is frequently detected in tumor tissue (39, 40, 55–58), and the incidence of intestinal tumorigenesis in both mice and humans can be reduced by pharmacological agents that inhibit Cox activity (41, 42, 59–64). A crucial role for *Cox-2* in tumorigenesis has been demonstrated by Oshima *et al.* (1996; Ref. 42), who found that intestinal polyposis in *Apc* mutant mice was markedly reduced by genetic ablation of *Cox-2*. However, *Cox-2* induction in response to Wnt proteins has not been demonstrated previously.

The mechanism by which Wnt-1 activates *Cox-2* transcription is unclear. Given that *COX-2* induction occurs in response to *Wnt-1* expression and *APC* mutation, both of which result in cytosolic β-catenin accumulation, our initial expectation was that the *Cox-2* promoter might be subject to direct regulation by β-catenin/Tcf complexes. The human *COX-2* promoter contains two potential Tcf-binding sites, although their overlap with the canonical TCF binding motif is only partial (ACTTTGATC and TCTTTGTAG compared with CCTTTGA/TA/TC; Ref. 27). One of these sites is not conserved in the murine *Cox-2* promoter, and the other lies outside the sequence presently reported for the mouse promoter. To investigate the mechanism of regulation, we have performed transient transfection assays using a human COX-2 promoter-luciferase reporter construct but thus far have been unable to detect increased reporter activity as a result of β-catenin overexpression. Thus, it is possible that *Cox-2* transcription may not be directly regulated by β-catenin/Tcf complexes but may be activated in *Wnt-1*-expressing cells by alternative transcription factors. We also cannot exclude the possibility that *Cox-2* induction is a more downstream or indirect consequence of Wnt signaling.

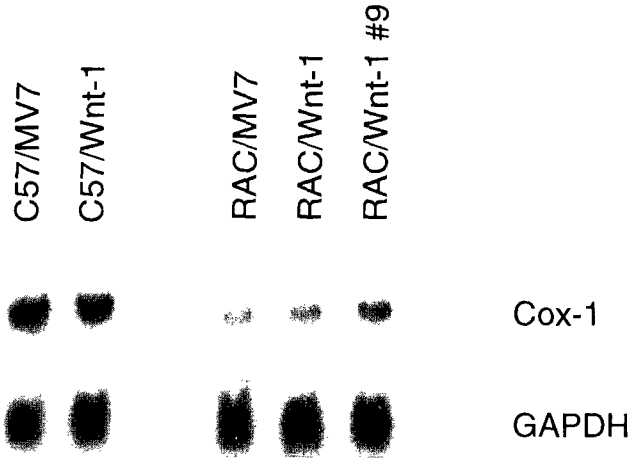

Fig. 6. Effect of *Wnt-1* expression on Cox-1 mRNA. RNA was prepared, and a Northern blot was generated as described in "Materials and Methods" using 20 μg of each RNA. The blot was probed sequentially with a murine *Cox-1* probe and a murine GAPDH probe. Cox-1 signals were quantitated using the program NIH Image and normalized to those obtained from GAPDH probing. Values obtained are expressed relative to the control MV7-infected cell line in each case. *C57/MV7*, 100%; *C57/Wnt-1*, 70%; *RAC/MV7*, 100%; *RAC/Wnt-1*, 118%; *RAC/Wnt-1 #9*, 163%.

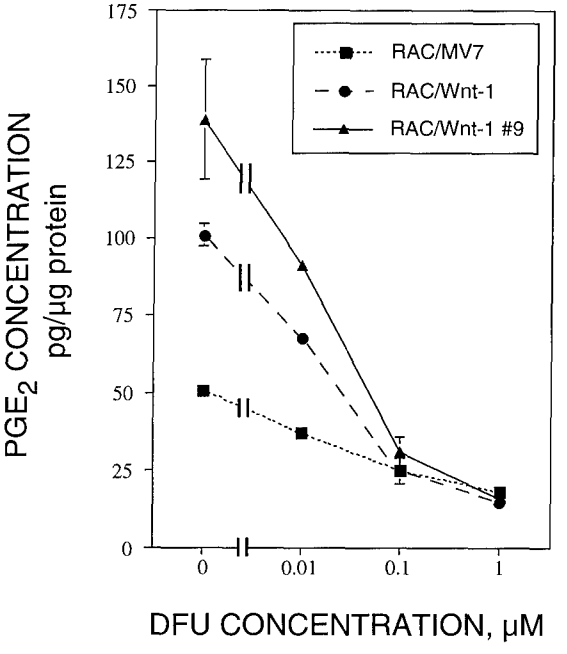

Fig. 7. Inhibition of PGE_2 production by a selective Cox-2 inhibitor DFU. Cells were treated with the indicated concentrations of DFU for 48 h. Culture medium was harvested and assayed for PGE_2 by enzyme immunoassay to determine spontaneous release of PGE_2. Results were normalized to μg of protein obtained from the cells after harvesting of the medium. Mean values of two replicates are shown; *bars*, spread.

Because *Wnt-1* is a mammary oncogene, our data suggest that *Cox-2* up-regulation might also contribute to mammary tumorigenesis. Consistent with this idea, *Cox-2* is expressed in ras- and virally transformed mammary cells, as well as in some human breast cancers and breast cancer cell lines (47, 65, 66). Interestingly, despite abundant evidence of the importance of Cox-2 during intestinal tumorigenesis, the precise mechanism by which Cox-2 contributes is unclear. Prostaglandin overproduction is likely to have multiple consequences. Prostaglandins can exert local immunosuppressive effects that could facilitate tumorigenesis (37, 67). Additionally changes in gene expression can occur because selected prostaglandins are ligands of the peroxisome proliferator-activated receptor γ (68). *Cox-2* induction in tumors may promote survival of cells otherwise destined to undergo apoptotic cell death; negative regulation of apoptosis by *Cox-2* overexpression has been demonstrated in intestinal epithelial cells (69). Recent data also demonstrate a role for Cox-2 in angiogenesis. Selective inhibition of Cox-2 reduces secretion of angiogenic factors from colon cancer cells, thereby suppressing *de novo* formation of endothelial tubules *in vitro* (70). Because of the pleiotropic consequences of *Cox-2* overexpression, it is difficult to predict what role Cox-2 might play in Wnt-1-induced mammary tumorigenesis. Transgenic mice that express *Wnt-1* ectopically in the mammary gland display extensive mammary hyperplasia at an early age and subsequently develop mammary adenocarcinomas stochastically after a latent period of several months (4). If Cox-2 contributes to Wnt-1-mediated tumorigenesis *in vivo*, it could do so either at the initial hyperplastic stage or by affecting progression to carcinoma. Our findings may also be pertinent to human breast cancer. Although expression of *WNT-1* itself in human mammary tissue has not been reported, several others members of the *WNT* gene family are overexpressed in human breast tumors relative to normal tissue (13–16). A number of Wnt proteins exhibit functional redundancy with Wnt-1, inducing stabilization of cytosolic β-catenin and morphological transformation of mammary cells (8, 10). Therefore, it is likely that some of the *WNT* genes overexpressed in human breast cancers may have transcriptional consequences similar to those of *Wnt-1*.

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REFERENCES

1. Nusse, R., and Varmus, H. E. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell, *31:* 99–109, 1982.
2. Peters, G., Brookes, S., Smith, R., and Dickson, C. Tumorigenesis by mouse mammary tumor virus: evidence for a common region for provirus integration in mammary tumors. Cell, *33:* 369–377, 1983.
3. Nusse, R., van Ooyen, A., Cox, D., Fung, Y. K. T., and Varmus, H. Mode of proviral activation of a putative mammary oncogene (*int*-1) on mouse chromosome 15. Nature (Lond.), *307:* 131–136, 1984.
4. Tsukamoto, A. S., Grosschedl, R., Guzman, R. C., Parslow, T., and Varmus, H. E. Expression of the *int*-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. Cell, *55:* 619–625, 1988.
5. Brown, A. M. C., Wildin, R. S., Prendergast, T. J., and Varmus, H. E. A retrovirus vector expressing the putative mammary oncogene *int*-1 causes partial transformation of a mammary epithelial cell line. Cell, *46:* 1001–1009, 1986.
6. Rijsewijk, F., van Deemter, L., Wagenaar, E., Sonnenberg, A., and Nusse, R. Transfection of the *int*-1 mammary oncogene in cuboidal RAC mammary cell line results in morphological transformation and tumorigenicity. EMBO J., *6:* 127–131, 1987.
7. Bradbury, J. M., Niemeyer, C. C., Dale, T. C., and Edwards, P. A. W. Alterations of the growth characteristics of the fibroblast cell line C3H 10T1/2 by members of the *Wnt* gene family. Oncogene, *9:* 2597–2603, 1994.
8. Wong, G. T., Gavin, B. J., and McMahon, A. P. Differential transformation of mammary epithelial cells by *Wnt* genes. Mol. Cell. Biol., *14:* 6278–6286, 1994.
9. Christiansen, J. H., Monkley, S. J., and Wainwright, B. J. Murine WNT11 is a secreted glycoprotein that morphologically transforms mammary epithelial cells. Oncogene, *12:* 2705–2711, 1996.
10. Shimizu, H., Julius, M. A., Giarré, M., Zheng, Z., Brown, A. M. C., and Kitajewski, J. Transformation by Wnt family proteins correlates with regulation of β-catenin. Cell Growth Differ., *8:* 1349–1358, 1997.
11. Young, C. S., Kitamura, M., Hardy, S., and Kitajewski, J. Wnt-1 induces growth, cytosolic β-catenin, and Tcf/Lef transcriptional activation in Rat-1 fibroblasts. Mol. Cell. Biol., *18:* 2474–2485, 1998.
12. Bafico, A., Gazit, A., Wu-Morgan, S. S., Yaniv, A., and Aaronson, S. A. Characterization of Wnt-1 and Wnt-2 induced growth alterations and signaling pathways in NIH3T3 fibroblasts. Oncogene, *16:* 2819–2825, 1998.
13. Huguet, E. L., McMahon, J. A., McMahon, A. P., Bicknell, R., and Harris, A. L. Differential expression of human *Wnt* genes 2, 3, 4, and 7B in human breast cell lines and normal and disease states of human breast tissue. Cancer Res., *54:* 2615–2621, 1994.
14. Iozzo, R. V., Eichstetter, I., and Danielson, K. G. Aberrant expression of the growth factor *Wnt-5A* in human malignancy. Cancer Res., *55:* 3495–3499, 1995.
15. Dale, T. C., Weber-Hall, S. J., Smith, K., Huguet, E. L., Jayatilake, H., Gusterson, B. A., Shuttleworth, G., O'Hare, M., and Harris, A. L. Compartment switching of *WNT*-2 expression in human breast tumors. Cancer Res., *56:* 4320–4323, 1996.
16. Bui, T. D., Rankin, J., Smith, K., Huguet, E. L., Ruben, S., Strachan, T., Harris, A. L., and Lindsay, S. A novel human *Wnt* gene, *WNT10B*, maps to 12q13 and is expressed in human breast carcinomas. Oncogene, *14:* 1249–1253, 1997.
17. Bergstein, I., and Brown, A. M. C. *WNT* genes and breast cancer. *In:* A. M. Bowcock (ed.), Breast Cancer: Molecular Genetics, Pathogenesis, and Therapeutics, pp. 181–198. Totowa, NJ: Humana Press, 1999.
18. van Ooyen, A., and Nusse, R. Structure and nucleotide sequence of the putative mammary oncogene *int*-1; proviral insertions leave the protein-encoding domain intact. Cell, *39:* 233–240, 1984.
19. Brown, A. M. C., Papkoff, J., Fung, Y. K., Shackleford, G. M., and Varmus, H. E. Identification of protein products encoded by the proto-oncogene *int*-1. Mol. Cell. Biol., *7:* 3971–3977, 1987.
20. Papkoff, J., Brown, A. M. C., and Varmus, H. E. The *int*-1 proto-oncogene products are glycoproteins that appear to enter the secretory pathway. Mol. Cell. Biol., *7:* 3978–3984, 1987.
21. Bradley, R. S., and Brown, A. M. C. A soluble form of Wnt-1 protein with mitogenic activity on mammary epithelial cells. Mol. Cell. Biol., *15:* 4616–4622, 1995.
22. Cadigan, K. M., and Nusse, R. Wnt signaling: a common theme in animal development. Genes Dev., *11:* 3286–3305, 1997.
23. Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. XTcf-3 transcription factor mediates β-catenin-induced axis formation in *Xenopus* embryos. Cell, *86:* 391–399, 1996.
24. Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. Functional interaction of β-catenin with the transcription factor LEF-1. Nature (Lond.), *382:* 638–642, 1996.
25. Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B. G., and Kemler, R. Nuclear localization of β-catenin by interaction with transcription factor LEF-1. Mech. Dev., *59:* 3–10, 1996.
26. Brunner, E., Peter, O., Schweizer, L., and Basler, K. *pangolin* encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in Drosophila. Nature (Lond.), *385:* 829–833, 1997.
27. van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., and Clevers, H. Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. Cell, *88:* 789–799, 1997.
28. Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S-C., Grosschedl, R., and Bienz, M. LEF-1, a nuclear factor coordinating signaling inputs from *wingless* and *decapentaplegic*. Cell, *88:* 777–787, 1997.
29. Porfiri, E., Rubinfeld, B., Albert, I., Hovanes, K., Waterman, M., and Polakis, P. Induction of a β-catenin-LEF-1 complex by wnt-1 and transforming mutants of β-catenin. Oncogene, *15:* 2833–2839, 1997.
30. Fagotto, F., Gluck, U., and Gumbiner, B. M. Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of β-catenin. Curr. Biol., *8:* 181–190, 1998.
31. Korinek, V., Barker, N., Willert, K., Molenaar, M., Roose, J., Wagenaar, G., Markman, M., Lamers, W., Destree, O., and Clevers, H. Two members of the Tcf family implicated in Wnt/β-catenin signaling during embryogenesis in the mouse. Mol. Cell. Biol., *18:* 1248–1256, 1998.
32. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, H. Constitutive transcriptional activation by a β-catenin-Tcf complex in APC$^{-/-}$ colon carcinoma. Science (Washington DC), *275:* 1784–1787, 1997.
33. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. Science (Washington DC), *275:* 1787–1790, 1997.
34. Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. Stabilization of β-catenin by genetic defects in melanoma cell lines. Science (Washington DC), *275:* 1790–1792, 1997.
35. Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., and Polakis, P. Regulation of intracellular β-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. Proc. Natl. Acad. Sci. USA, *92:* 3046–3050, 1995.
36. Sparks, A. B., Morin, P. J., Vogelstein, B., and Kinzler, K. W. Mutational analysis of the APC/β-catenin/Tcf pathway in colorectal cancer. Cancer Res., *58:* 1130–1134, 1998.

37. Subbaramaiah, K., Zakim, D., Weksler, B. B., and Dannenberg, A. J. Inhibition of cyclooxygenase: a novel approach to cancer prevention. Proc. Soc. Exp. Biol. Med., *216:* 201–210, 1997.
38. Herschman, H. R. Prostaglandin synthase 2. Biochim. Biophys. Acta, *1299:* 125–140, 1996.
39. Kargman, S. L., O'Neill, G. P., Vickers, P. J., Evans, J. F., Mancini, J. A., and Jothy, S. Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. Cancer Res., *55:* 2556–2559, 1995.
40. Williams, C. S., Luongo, C., Radhika, A., Zhang, T., Lamps, L. W., Nanney, L. B., Beauchamp, R. D., and DuBois, R. N. Elevated cyclooxygenase-2 levels in *Min* mouse adenomas. Gastroenterology, *111:* 1134–1140, 1996.
41. Boolbol, S. K., Dannenberg, A. J., Chadburn, A., Martucci, C., Guo, X., Ramonetti, J. T., Abreu-Goris, M., Newmark, H. L., Lipkin, M. L., DeCosse, J. J., and Bertagnolli, M. M. Cyclooxygenase-2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis. Cancer Res., *56:* 2556–2560, 1996.
42. Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. Suppression of intestinal polyposis in $Apc^{\Delta 716}$ knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell, *87:* 803–809, 1996.
43. Vaidya, A. B., Lasfargues, E. Y., Sheffield, J. B., and Coutinho, W. G. Murine mammary tumor virus (MMuTV) infection of an epithelial cell line established from C57BL/6 mouse mammary glands. Virology, *90:* 12–22, 1978.
44. Ramakrishna, N. R., and Brown, A. M. C. *Wingless*, the *Drosophila* homolog of the proto-oncogene *Wnt*-1, can transform mouse mammary epithelial cells. Dev. *119, Suppl.:* 95–103, 1993.
45. Sonnenberg, A., van Balen, P., Hilgers, J., Schuuring, E., and Nusse, R. Oncogene expression during progression of mouse mammary tumor cells: activity of a proviral enhancer and the resulting expression of int-2 is influenced by the state of differentiation. EMBO J., *6:* 121–125, 1987.
46. Giarré, M., Semënov, M. V., and Brown, A. M. C. WNT signaling stabilizes the dual function protein β-catenin in diverse cell types. Ann. NY Acad. Sci., *857:* 43–55, 1998.
47. Subbaramaiah, K., Telang, N., Ramonetti, J. T., Araki, R., DeVito, B., Weksler, B. B., and Dannenberg, A. J. Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells. Cancer Res., *56:* 4424–4429, 1996.
48. Church, G. M., and Gilbert, W. Genomic sequencing. Proc. Natl. Acad. Sci. USA, *81:* 1991–1995, 1984.
49. Papkoff, J., Rubinfeld, B., Schryver, B., and Polakis, P. Wnt-1 regulates free pools of catenins and stabilizes APC-catenin complexes. Mol. Cell. Biol., *16:* 2128–2134, 1996.
50. Oshima, T., Yoshimoto, T., Yamamoto, S., Kumegawa, M., Yokoyama, C., and Tanabe, T. cAMP-dependent induction of fatty acid cyclooxygenase mRNA in mouse osteoblastic cells (MC3T3–E1). J. Biol. Chem., *266:* 13621–13626, 1991.
51. Hamasaki, Y., Kitzler, J., Hardman, R., Nettesheim, P., and Eling, T. E. Phorbol ester and epidermal growth factor enhance the expression of two inducible prostaglandin H synthase genes in rat tracheal epithelial cells. Arch. Biochem. Biophys., *304:* 226–234, 1993.
52. Nanayama, T., Hara, S., Inoue, H., Yokoyama, C., and Tanabe, T. Regulation of two isozymes of prostaglandin endoperoxide synthase and thromboxane synthase in human monoblastoid cell line U937. Prostaglandins, *49:* 371–382, 1995.
53. Murakami, M., Matsumoto, R., Urade, Y., Austen, K. F., and Arm, J. P. c-kit ligand mediates increased expression of cytosolic phospholipase A_2, prostaglandin endoperoxide synthase-1, and hematopoietic prostaglandin D_2 synthase and increased IgE-dependent prostaglandin D_2 generation in immature mouse mast cells. J. Biol. Chem., *270:* 3239–3246, 1995.
54. Riendeau, D., Percival, M. D., Boyce, S., Brideau, C., Charleson, S., Cromlish, W., Ethier, D., Evans, J., Falgueryet, J-P., Ford-Hutchinson, A. W., Gordon, R., Greig, G., Gresser, M., Guay, J., Kargmann, S., Leger, S., Mancini, J. A., O'Neill, G., Ouellet, M., Rodger, I. W., Therien, M., Wang, Z., Webb, J. K., Wong, E., Xu, L., Young, R. N., Zamboni, R., Prasit, P., and Chan, C-C. Biochemical and pharmacological profile of a tetrasubstituted furanone as a highly selective COX-2 inhibitor. Br. J. Pharmacol., *121:* 105–117, 1997.
55. Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S., and DuBois, R. N. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology, *107:* 1183–1188, 1994.
56. Sano, H., Kawahito, Y., Wilder, R. L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M., and Hla, T. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res., *55:* 3785–3789, 1995.
57. Kutchera, W., Jones, D. A., Matsunami, N., Groden, J., McIntyre, T. M., Zimmerman, G. A., White, R. L., and Prescott, S. M. Prostaglandin H synthase 2 is expressed abnormally in human colon cancer: evidence for a transcriptional effect. Proc. Natl. Acad. Sci. USA, *93:* 4816–4820, 1996.
58. DuBois, R. N., Radhika, A., Reddy, B. S., and Entingh, A. J. Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors. Gastroenterology, *110:* 1259–1262, 1996.
59. Smalley, W. E., and DuBois, R. N. Colorectal cancer and non steroidal anti-inflammatory drugs. Adv. Pharmacol., *39:* 1–20, 1997.
60. Giardiello, F. M., Hamilton, S. R., Krush, A. J., Piantadosi, S., Hylind, L. M., Celano, P., Booker, S. V., Robinson, C. R., and Offerhaus, G. J. A. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. N. Engl. J. Med., *328:* 1313–1316, 1993.
61. Reddy, B. S., Rao, C. V., and Seibert, K. Evaluation of cyclooxygenase-2 inhibitor for potential chemopreventive properties in colon carcinogenesis. Cancer Res., *56:* 4566–4569, 1996.
62. Yoshimi, N., Kawabata, K., Hara, A., Matsunaga, K., Yamada, Y., and Mori, H. Inhibitory effect of NS-398, a selective cyclooxygenase-2 inhibitor, on azoxymethane-induced aberrant crypt foci in colon carcinogenesis of F344 rats. Jpn. J. Cancer Res., *88:* 1044–1051, 1997.
63. Nakatsugi, S., Fukutake, M., Takahashi, M., Fukuda, K., Isoi, T., Taniguchi, Y., Sugimura, T., and Wakabayashi, K. Suppression of intestinal polyp development by nimesulide, a selective cyclooxygenase-2 inhibitor, in Min mice. Jpn. J. Cancer Res., *88:* 1117–1120, 1997.
64. Kawamori, T., Rao, C. V., Seibert, K., and Reddy, B. S. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. Cancer Res., *58:* 409–412, 1998.
65. Liu, X-H., and Rose, D. P. Differential expression and regulation of cyclooxygenase-1 and -2 in two human breast cancer cell lines. Cancer Res., *56:* 5125–5127, 1996.
66. Parrett, M. L., Harris, R. E., Joarder, F. S., Ross, M. S., Clausen, K. P., and Robertson, F. M. Cyclooxygenase-2 gene expression in human breast cancer. Int. J. Oncol., *10:* 503–507, 1997.
67. Huang, M., Stolina, M., Sharma, S., Mao, J. T., Zhu, L., Miller, P. W., Wollman, J., Herschman, H., and Dubinett, S. M. Non-small cell lung cancer cyclooxygenase-2-dependent regulation of cytokine balance in lymphocytes and macrophages: up-regulation of interleukin 10 and down-regulation of interleukin 12 production. Cancer Res., *58:* 1208–1216, 1998.
68. Yu, K., Bayona, W., Kallen, C. B., Harding, H. P., Ravera, C. P., McMahon, G., Brown, M., and Lazar, M. A. Differential activation of peroxisome proliferator-activated receptors by eicosanoids. J. Biol. Chem., *270:* 23975–23983, 1995.
69. Tsujii, M., and DuBois, R. N. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell, *83:* 493–501, 1995.
70. Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M., and DuBois, R. N. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell, *93:* 705–716, 1998.
71. Bradley, R. S., and Brown, A. M. C. The proto-oncogene *int*-1 encodes a secreted protein associated with the extracellular matrix. EMBO J., *9:* 1569–1575, 1990.

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PEA3 Is Up-regulated in Response to Wnt1 and Activates the Expression of Cyclooxygenase-2*

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Louise R. Howe‡§¶, Howard C. Crawford‖, Kotha Subbaramaiah§ ‡‡, John A. Hassell§§ ¶¶, Andrew J. Dannenberg§ ‡‡, and Anthony M. C. Brown‡§**

From the ‡Department of Cell Biology and Anatomy, Weill Medical College of Cornell University, New York, New York 10021, the §Strang Cancer Research Laboratory at the Rockefeller University, New York, New York 10021, the ‖Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232, the ‡‡Department of Medicine, Weill Medical College of Cornell University, New York, New York 10021, and the §§Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario L86 4K1, Canada

The inducible prostaglandin synthase cyclooxygenase-2 (COX-2) is aberrantly expressed in intestinal tumors resulting from *APC* mutation, and is also transcriptionally up-regulated in mouse mammary epithelial cells in response to *Wnt1* expression. β-Catenin stabilization is a consequence of both *APC* mutation and Wnt signaling. We have previously observed coordinate regulation of the matrilysin promoter by β-catenin and Ets family transcription factors of the PEA3 subfamily. Here we show that while β-catenin only weakly activates the *COX-2* promoter, PEA3 family transcription factors are potent activators of *COX-2* transcription. Consistent with this, *PEA3* is up-regulated in *Wnt1*-expressing mouse mammary epithelial cells, and PEA3 factors are highly expressed in tumors from *Wnt1* transgenic mice, in which *Cox-2* is also up-regulated. Promoter mapping experiments suggest that the NF-IL6 site in the *COX-2* promoter is important for mediating PEA3 responsiveness. The NF-IL6 site is also important for *COX-2* transcription in some colorectal cancer lines (Shao, J., Sheng, H., Inoue, H., Morrow, J. D., and DuBois, R. N. (2000) *J. Biol. Chem.* 275, 33951–33956), and PEA3 factors are highly expressed in colorectal cancer cell lines. Therefore, we speculate that PEA3 factors may contribute to the up-regulation of *COX-2* expression resulting from both *APC* mutation and *Wnt1* expression.

Wnt1 is a mammary oncogene that encodes a secreted signaling factor. Targeted expression of *Wnt1* in murine mammary glands results in epithelial hyperplasia with subsequent carcinoma formation (1). Wnt1 signaling leads to stabilization of a cytosolic pool of β-catenin, and consequently transcriptional activation by β-catenin·TCF[1] complexes (2, 3). Additionally, β-catenin may induce TCF-independent transcription (4–6). Inappropriate activation of the Wnt signaling pathway has been detected in numerous tumors, arising as a consequence of *Wnt* gene misexpression, *APC* mutation, or mutation of other components of the pathway such as axin and β-catenin itself (7). Multiple transcriptional targets of Wnt signaling have now been identified, some of which are likely to contribute to tumorigenesis. Of these, several have been demonstrated to be directly activated by β-catenin, including cyclin D1, c-*myc*, matrilysin, and peroxisome proliferator activated receptor δ (8–12). In addition, we have shown transcriptional up-regulation of *Cox-2* in *Wnt1*-expressing mouse mammary epithelial cells (13), but did not determine whether this was due to direct regulation of the *Cox-2* promoter by β-catenin.

Cox-2, the inducible isoform of prostaglandin synthase, is aberrantly expressed in human colorectal cancers, and also in tumors from mouse colorectal cancer models carrying germline *Apc* mutations (14–18). Additionally, *COX-2* overexpression has now been detected in multiple human cancers including those of the skin, head and neck, lung, breast, and stomach (19–24). Strikingly, *COX-2* overexpression in murine mammary gland is sufficient to induce tumorigenesis (25). Thus, considerable interest is focused on COX-2 as a potential therapeutic target for the prevention or treatment of cancer. Both genetic ablation and pharmacological inhibition of COX-2 have resulted in reduced tumorigenesis in several animal cancer models (26–30), and selective COX-2 inhibitors have also proved effective in reducing the number of colorectal polyps in familial adenomatous polyposis patients (31). Several mechanisms have been proposed to account for the role of COX-2 in tumorigenesis. *Cox-2* overexpression in epithelial cells is associated with enhanced invasiveness and suppression of apoptosis (25, 32, 33). Prostaglandin overproduction is likely to have pleiotropic consequences including stimulation of proliferation and local immunosuppressive effects that could facilitate tumorigenesis. Recent data also demonstrate a role for Cox-2 in angiogenesis (34–39).

Modulation of COX-2 protein levels can be achieved via multiple mechanisms, including transcriptional activation, mRNA stabilization, and altered COX-2 protein stability. We have previously demonstrated transcriptional up-regulation of *Cox-2* in response to *Wnt1* expression in mouse mammary epithelial cells (13). The goal of the current study was to elu-

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¶ To whom correspondence should be addressed: Strang Cancer Research Laboratory, Rockefeller University, Box 231, 1230 York Ave., New York, NY 10021. Tel.: 212-734-0567; Fax: 212-472-9471; E-mail: lrhowe@med.cornell.edu.

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[1] The abbreviations used are: TCF, T cell factor; C/EBP, CCAAT/enhancer-binding protein; COX, cyclooxygenase; CRE, cyclic AMP response element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LIP, liver-enriched inhibitory protein; MG, mammary glands; NF-IL6, nuclear factor interleukin-6; NF-κB, nuclear factor-κB; MOPS, 4-morpholinopropanesulfonic acid.

cidate the mechanism(s) underlying Wnt1-mediated induction of *Cox-2*. Given that *COX-2* is also up-regulated in intestinal tumors resulting from *APC* mutation, we initially hypothesized that β-catenin might regulate the *COX-2* promoter. Here we examine the effect of both β-catenin and Ets family transcription factors of the PEA3 subfamily on *COX-2* promoter activity.

EXPERIMENTAL PROCEDURES

Cell Culture—Generation, characterization, and culture of the control and *Wnt1*-expressing mouse mammary epithelial cell lines have been previously described (13). C57/MV7 and RAC/MV7 are control populations infected with MV7 retrovirus, while C57/Wnt-1 and RAC/Wnt-1 are *Wnt1*-expressing populations, and RAC/Wnt-1 #9 is a clonal subline selected for high level *Wnt1* expression. Identical culture conditions were used for the parental cell line C57MG. 293 human embryonic kidney cells were grown in Dulbecco's modified Eagle's medium (4.5 g/liter D-glucose) containing 10% fetal bovine serum (Life Technologies, Inc.), 100 units/ml penicillin, and 100 μg/ml streptomycin.

Cell Transfection and Luciferase Assays—293 cells were transfected using LipofectAMINE (Life Technologies), according to the manufacturer's instructions. Briefly, cells were seeded in 24-well plates at 1×10^5 cells/well. 20 h after cell seeding, 6 wells were transfected for 4 h using a transfection mixture consisting of 1.2 ml of serum-free Dulbecco's modified Eagle's medium, 3.6 μl of LipofectAMINE, and 2.2 μg of total plasmid DNA (including 0.2 μg of pRL-TK). Where necessary "empty" vectors were included to maintain constant amounts of DNA. Lysates were prepared 48 h after transfection, and Firefly and Renilla luciferase activities were measured using a Dual Luciferase Reagent kit (Promega) and a Monolight 2010 luminometer (Analytical Luminescence Laboratory). Firefly activity was normalized to the Renilla activity, and results were expressed as percentage of control activity. To transfect 293 cells for protein analysis or RNA preparation, cells were plated at 5×10^6 cells per 10-cm plate, and transfections scaled-up proportionally to surface area. Similar conditions were used to transfect C57MG cells, except that cells were plated at 2.5×10^4 cells/well, and 5.4 μl of LipofectAMINE was used for transfection.

Mammary Tissues and Tumors—A breeding colony of *Wnt1* transgenic mice (1) was maintained by crossing *Wnt1* transgenic B6/SJL males (obtained from the Jackson Laboratory) with strain-matched females. Mice were genotyped by polymerase chain reaction analysis of tail-tip DNA. The primers used were: 5′-CCAGAACACAGCATGGCTTCCAACG-3′ and 5′-ACTCCACACAGGCATAGAGTGTCTGC-3′. These primers amplify a 425-base pair fragment present in the transgene, but not in the endogenous *Wnt1* gene. *Wnt1* transgenic animals were sacrificed when tumors were 1 cm in diameter, and wild type littermates were simultaneously sacrificed. Tumors and mammary glands were snap-frozen in liquid nitrogen and stored at −80 °C prior to use.

Protein Analysis—Transfected 293 cells were lysed, and lysates analyzed for expression of Myc-epitope-tagged proteins and ERK2, using 9E10 monoclonal and 122 polyclonal antibodies, respectively, as previously described (40). Cox-2 protein in mammary glands and tumors was assayed using a coupled immunoprecipitation/immunoblotting assay. 10 mg of mammary gland or tumor tissue was sonicated in 1 ml of RIPA buffer, and the sonicate was centrifuged at 10,000 × *g* for 10 min at 4 °C. The resulting supernatant was precleared by incubation at 4 °C with goat IgG, rabbit IgG, and Protein-G-PLUS agarose (Santa Cruz Biotechnology Inc.). Cox-2 protein was immunoprecipitated by incubation at 4 °C for 1 h with 10 μl each of rabbit polyclonal and goat polyclonal anti-Cox-2 antibodies (Oxford Biomedical Research, Inc. and Santa Cruz Biotechnology Inc., respectively), followed by an additional 16-h incubation after addition of 20 μl of Protein A-agarose. Beads were recovered by centrifugation (5 min, 3,000 × *g*, 4 °C), and washed 4 times with RIPA buffer prior to resuspension in Laemmli sample buffer. Cox-2 protein was detected by Western blotting after running the immunoprecipitates on SDS-polyacrylamide gels as described previously (13).

RNA Preparation and Northern Blotting—RNA was prepared from confluent cells and from mammary glands and tumors using RNAzol B (Tel-Test, Inc.) according to the manufacturer's instructions. Frozen tissues were finely minced prior to homogenization in RNAzol B. Northern blot analysis was performed as described previously using MOPS/formaldehyde gels (13).

Plasmids—Northern probes used were as follows: human COX-2 (S. M. Prescott, University of Utah, Salt Lake City, UT), murine PEA3 (41), murine ERM (42), murine ER81 (42), and GAPDH (13). The *COX-2* promoter reporter construct COX-2-LUC contained nucleotides −1432/+59 of the human *COX-2* promoter linked to luciferase (43). In addition, the following truncated *COX-2* promoter constructs were used: −327/+59, −220/+59, −124/+59, and −52/+59 (43). Constructs were also utilized in which mutations had been introduced into the −327/+59 backbone. KBM, ILM and CRM have mutagenized NF-κB (−223/−214), NF-IL6 (−132/−124), and CRE (−59/−53) sites, respectively (43). The stromelysin-1 promoter construct p754TR-Luc (42) was used to compare activation by Ets factors with that of the COX-2 promoter. p754TR-Luc was co-transfected with expression vectors encoding Ets factors plus c-Jun, since Ets factors alone were insufficient to induce stromelysin-1 promoter activity. The TOPFLASH vector, an artificial β-catenin·TCF-responsive promoter reporter (44), was used to confirm that overexpressed β-catenin could drive transcription. The following

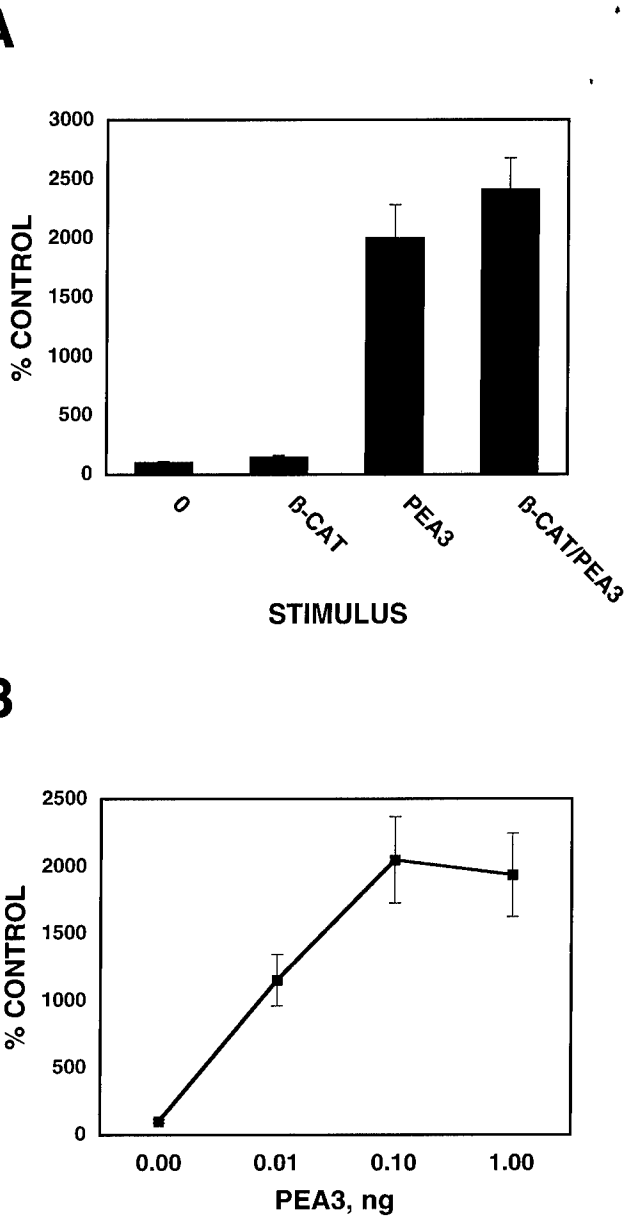

FIG. 1. **Effects of β-catenin and PEA3 on *COX-2* promoter regulation.** *A*, effect of PEA3 and β-catenin on *COX-2* promoter activity. 293 cells were transfected with combinations of expression vectors encoding β-catenin and PEA3, together with a *COX-2* promoter luciferase reporter construct (COX-2-LUC), and with pRL-TK as an internal control. Luciferase activities were measured as described under "Experimental Procedures." Results shown are the mean + S.D. of six replicates from a representative experiment, expressed relative to activity in control cells transfected with empty expression vectors. *B*, PEA3 dose dependence of *COX-2* promoter activation. 293 cells were transfected with increasing amounts of PEA3 expression vector, plus COX-2-LUC and pRL-TK, and luciferase assays were performed as described above. Results shown are the mean ± S.D. of 6 replicates.

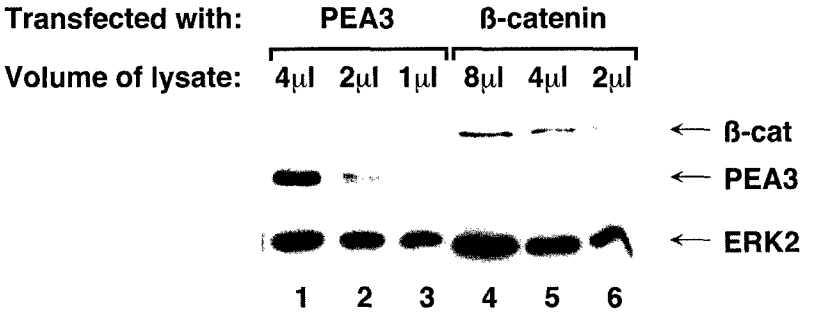

FIG. 2. **Comparison of β-catenin and PEA3 expression.** 293 cells were transfected with expression vectors encoding β-catenin or PEA3, and lysates prepared as described under "Experimental Procedures." Equivalent amounts of each expression vector were used as in the experiments shown in Figs. 1*A* and 4. To compare the relative expression levels of β-catenin and PEA3, varied amounts of each lysate were electrophoresed on the same polyacrylamide gel, and analyzed by Western blotting sequentially with the anti-Myc epitope antibody 9E10 (*upper panel*) and the anti-ERK2 antibody 122 (*lower panel*). 9E10 detected both PEA3 (*lanes 1–3*) and β-catenin (β-cat; *lanes 4–6*), since both are Myc-epitope-tagged. Anti-ERK2 antibody was used as a loading control. Comparison of *lane 5* with *lanes 2* and *3* suggests that β-catenin is present at 2–4-fold lower levels than PEA3.

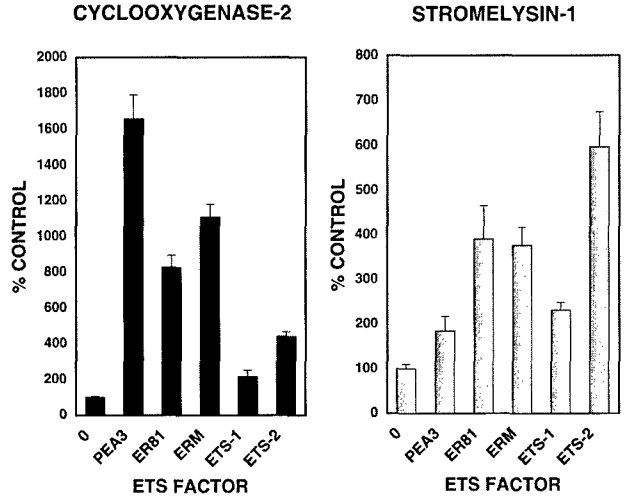

FIG. 3. **The *COX-2* promoter is selectively activated by PEA3 factors.** 293 cells were transfected with expression vectors encoding PEA3, ER81, ERM, ETS-1, or ETS-2, together with COX-2-LUC and pRL-TK. In parallel, cells were transfected with the Ets expression vectors, pRL-TK, a stromelysin promoter luciferase reporter construct (p754TR-Luc), and a c-Jun expression vector. Luciferase assays were performed as described above. Results shown are the mean + S.D. of six replicates.

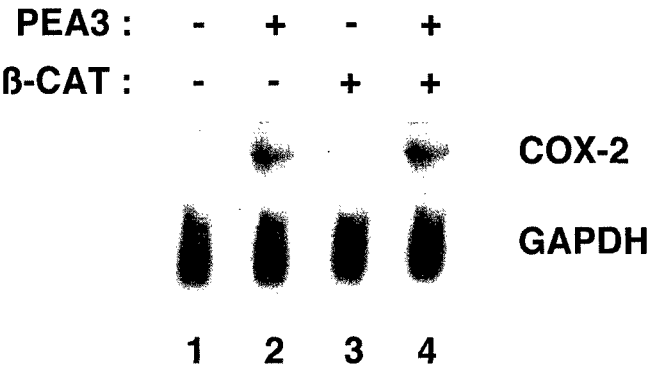

FIG. 4. **Activation of the endogenous *COX-2* promoter by *PEA3* overexpression.** 293 cells were transfected with combinations of expression vectors encoding β-catenin and PEA3, and total RNA was prepared from cells 48 h after transfection. 20 µg of each RNA was analyzed by Northern blotting as described under "Experimental Procedures." The blot was hybridized sequentially with probes for COX-2 and GAPDH.

expression vectors were used: pMT23β-catenin (myc-tagged; 45), pCANmycPEA3 (42), pcDNA-ER81 (42), pCANmycERM (42), Ets-1 (S. Hiebert, Vanderbilt University, Nashville, TN), pSG5-Ets-2 (D. Watson, Medical University of South Carolina, Charleston, SC), pCMX-c-jun (R. Wisdom, Vanderbilt University), and pCMV-LIP, encoding dominant negative C/EBPβ (46, 47). In addition, expression vectors encoding C/EBPα, C/EBPβ, and C/EBPδ were obtained from S. McKnight (University of Texas, Southwestern, TX).

RESULTS

Transient transfections were performed to determine whether the *COX-2* promoter was susceptible to regulation by β-catenin. The 293 human embryonic kidney cell line was selected for these experiments based on ease of transfection (4). 293 cells were co-transfected with a β-catenin expression vector and with COX-2-LUC, a luciferase reporter construct containing 1.4 kilobase pairs of the human *COX-2* promoter (43). In addition, we tested the effect of the Ets family transcription factor PEA3 since we had previously observed synergistic activation of another target gene, matrilysin, by PEA3 and β-catenin (42). Overexpression of β-catenin stimulated the activity of TOPFLASH, an artificial β-catenin/TCF-responsive promoter reporter (44), by 5–10-fold (data not shown). However, we observed only very weak activation of the *COX-2* promoter construct COX-2-LUC by β-catenin (Fig. 1*A*). The mean increase observed in six experiments in response to β-catenin was 28% ($p < 0.03$). In contrast, PEA3 activated COX-2-LUC up to 20-fold (Fig. 1, *A* and *B*). Together PEA3 and β-catenin elicited a greater than additive response in some experiments (Fig. 1*A*), although the observed trend was not statistically significant.

One potential explanation for the contrasting potencies of PEA3 and β-catenin in activating the *COX-2* promoter could be different expression levels of the two proteins. To directly compare expressions levels, lysates were prepared from 293 cells transfected with expression vectors for PEA3 or β-catenin (both of which were Myc epitope-tagged) and analyzed by Western blotting with anti-Myc epitope antibody 9E10. On this basis, PEA3 was expressed at 2–4-fold higher levels than β-catenin (Fig. 2). However, transfection of 50-fold less PEA3 than used in this experiment was sufficient to potently activate the *COX-2* promoter (Fig. 1*B*), and it therefore seems unlikely that this small difference in expression levels is sufficient to explain the differential responsiveness of the *COX-2* promoter to β-catenin and PEA3.

We also examined the response of the *COX-2* promoter to the Ets factors Ets-1 and Ets-2, in comparison with the PEA3 subfamily members PEA3, ER81, and ERM. Of these, PEA3 was the most potent activator of the *COX-2* promoter (Fig. 3). ER81 and ERM were also capable of inducing significant activation, in contrast with the weaker responses elicited by Ets-1 and Ets-2. It was not possible to compare expression levels of the various factors directly, since the cDNAs were not uniformly epitope-tagged. However, since Ets-2 caused much stronger activation of the stromelysin-1 promoter than did

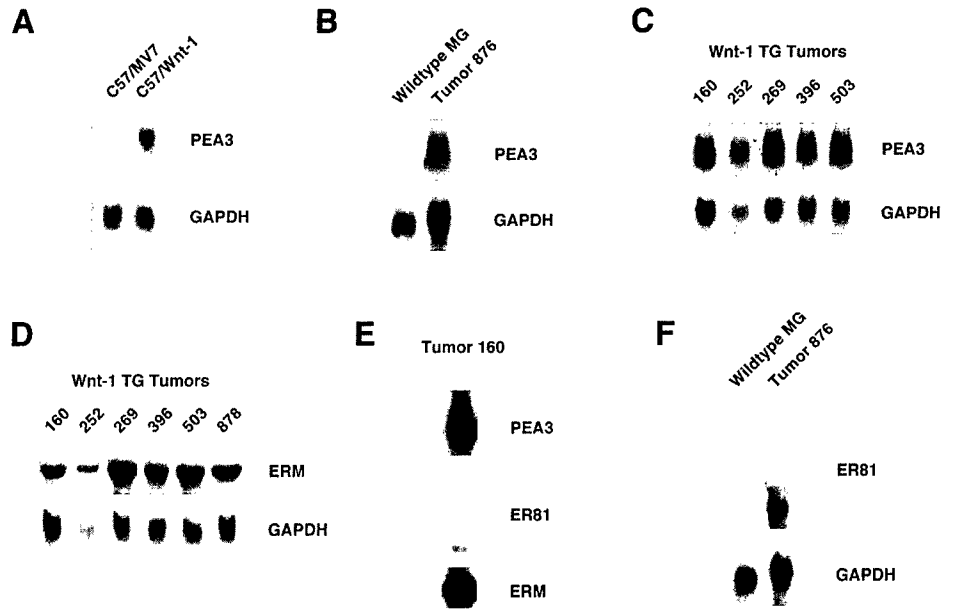

FIG. 5. **PEA3 factor expression analysis.** *A, PEA3* expression is up-regulated in C57MG cells expressing *Wnt1*. Total RNA was prepared from C57/MV7 and C57/Wnt-1 cells and 20 μg analyzed by Northern blotting as described under "Experimental Procedures." The blot was hybridized sequentially with probes for PEA3 and GAPDH. *B,* comparison of *PEA3* expression in mammary glands and tumor tissue. Total RNA was prepared from a mammary carcinoma from a *Wnt1* transgenic mouse. RNA was also prepared from the MG of an age-matched wild type littermate. 20 μg of each RNA was analyzed by Northern blotting, and hybridized sequentially with probes for PEA3 and GAPDH. A high level of *PEA3* expression was detected in the tumor. Longer exposures revealed much lower *PEA3* expression in wild type MG (not shown). *C, PEA3* expression in tumors from *Wnt1* transgenic mice. Total RNA was prepared from mammary carcinomas from five *Wnt1* transgenic mice and analyzed by Northern blotting for *PEA3* expression as in *B*. High level expression was observed in all tumors examined. *D, ERM* expression in tumors from *Wnt1* transgenic mice. RNA from 6 mammary tumors was analyzed by Northern blotting for *ERM* expression by sequential probing with an ERM probe and a GAPDH probe. High level expression was observed in all tumors examined. No ERM transcript was detectable in wild type MG (not shown). *E,* comparison of *PEA3, ER81,* and *ERM* expression in a single *Wnt1* transgenic tumor. RNA from a single tumor was subjected to Northern blotting and probed in parallel with probes for PEA3, ER81, and ERM. All probes were of approximately equal specific activity, and exposures to film were of the same duration, thus allowing an approximate comparison of relative expression levels. *ER81* was expressed at a much lower level (in tumor 160 and in 6 other tumors tested) than were *PEA3* and *ERM*. One major and two minor ER81 transcripts were detected. GAPDH probing confirmed equal RNA loading (not shown). *F,* comparison of *ER81* expression in tumor tissue and wild type mammary glands. 20 μg of total RNA from wild type MG and from tumor from a *Wnt1* transgenic mouse was analyzed by Northern blotting, with sequential probing with an ER81 probe and a GAPDH probe. No *ER81* expression was detectable in wild type MG, but a distinct signal was detected in *Wnt1* transgenic tumor tissue. Similar *ER81* expression was detected in all 7 tumors tested (not shown).

PEA3 in the same experiment, we conclude that the *COX-2* promoter is preferentially responsive to PEA3 family members (Fig. 3). Strikingly, PEA3 alone was also sufficient to activate transcription of the endogenous *COX-2* gene. Transient transfection of PEA3 into 293 cells caused accumulation of *COX-2* transcript (Fig. 4). In contrast, β-catenin alone did not stimulate significant transcription of the endogenous *COX-2* gene, nor did it enhance the response to PEA3, consistent with the data obtained using the *COX-2* promoter reporter construct (Fig. 1*A*).

Since we had previously observed *Cox-2* up-regulation in *Wnt1*-expressing cell lines (13), we were interested to determine whether PEA3 factors could play a role in this up-regulation. In particular, we had observed significantly increased *Cox-2* transcript in response to Wnt1 in C57MG cells, a mouse mammary epithelial cell line which undergoes Wnt1-induced morphological transformation (48). Therefore *PEA3* transcripts were measured in C57MG-derived cell lines, and also in mammary tumors from *Wnt1* transgenic mice. We observed up-regulated *PEA3* expression in C57MG cells in response to *Wnt1* expression (Fig. 5*A*). This observation is consistent with the *Cox-2* up-regulation observed in these cells, and suggests a role for PEA3 in *Cox-2* promoter regulation in these cells. To test this, the effects of PEA3 and β-catenin on *COX-2* promoter activity in C57MG cells were compared. *PEA3* overexpression increased *COX-2* promoter activity to 205% ($p < 0.03$; Fig. 6). A smaller degree of activation was observed in response to β-catenin in several experiments, but was not statistically significant. These experiments were limited by the poor transfection efficiency associated with C57MG cells (4). Nevertheless, our data suggest that, both in 293 and C57MG cells (Figs. 1 and 6), PEA3 is a more potent activator of the *COX-2* promoter than β-catenin.

In addition to being up-regulated in *Wnt1*-expressing C57MG cells, *PEA3* was highly expressed in all tumors examined from *Wnt1* transgenic mice (Figs. 5, *B* and *C*), contrasting with the very low expression level in normal virgin mammary gland (41) (Fig. 5*B*). *ERM* was also highly expressed in all *Wnt1* tumors tested, while no expression was detected in wild type mammary gland (Fig. 5*D*; data not shown). Comparison of all three factors revealed that, in contrast to *PEA3* and *ERM*, *ER81* was expressed at a relatively low level in the tumors (Fig. 5*E*, data not shown), albeit at higher levels than in virgin mammary gland (Fig. 5*F*).

Our observation of high level expression of PEA3 factors in tumors from *Wnt1* transgenic mice, coupled with the demonstration that PEA3 activates the *COX-2* promoter, prompted us to test whether *Cox-2* was also up-regulated in *Wnt1*-expressing mammary tumors. Levels of Cox-2 protein were compared in normal mammary glands from wild type mice and in tumors from *Wnt1* transgenic mice. We observed a significant increase in Cox-2 protein in the three tumors tested compared with virtually undetectable basal levels in wild type mammary gland (Fig. 7). These data suggest that increased expression of PEA3 factors may contribute to *Cox-2* up-regulation in these tumors.

In order to map the PEA3-responsive elements in the *COX-2* promoter, a range of promoter reporter constructs were used in

which deletions or site-specific mutations had been introduced (43). Progressive truncation of the promoter sequentially deletes binding sites for NF-κB, NF-IL6, and finally the CRE (Fig. 8*A*). No significant diminution in PEA3 responsiveness was observed until residues −220 to −125 were deleted (Fig. 8*B*). The −124/+59 construct was virtually unresponsive to PEA3, suggesting that the PEA3 response element(s) lie between −220 and −125. Since an NF-IL6-binding site is present in this region of the promoter, we next tested the responsiveness to PEA3 of various site-specific promoter mutants, including the ILM construct in which the NF-IL6 site is mutagenized. This experiment was primarily intended to rule out involvement of this site in the PEA3 response. However, to our surprise, we found that mutation of the NF-IL6 site completely and specifically abolished both basal and PEA3-stimulated *COX-2* promoter activity (Fig. 9). In contrast, mutation of the NF-κB site had no effect on PEA3 responsiveness. Mutation of the CRE site reduced both basal and PEA-3-stimulated activity, such that the index of stimulation exhibited by the CRM construct was not reduced relative to the wild type construct. Collectively, these data strongly implicate the NF-IL6 site in mediating PEA3 responsiveness. Since we have previously found mutation of the NF-IL6 site to have little effect on *COX-2* promoter responsiveness to the lipid ceramide (49), we believe these data to reflect a specific involvement of the NF-IL6 site in mediating PEA3 responsiveness.

The NF-IL6 site is a consensus binding site for transcription factors of the C/EBP family. Several C/EBP factors have been identified (50). C/EBPα is generally associated with differentiation, while C/EBPβ and C/EBPδ are primarily implicated in mediating gene activation during inflammation and cell proliferation. Our data demonstrating the importance of the NF-IL6 site for PEA3 responsiveness suggested that C/EBP factors might be involved in PEA3-mediated activation of the *COX-2* promoter. To test this hypothesis we used several C/EBP expression constructs, including a dominant negative variant, LIP (liver-enriched inhibitory protein). Consistent with previous observations in other cell types (43, 51), both C/EBPα and C/EBPδ stimulated *COX-2* promoter activity, while C/EBPβ had little effect on basal promoter activity (Fig. 10*A*). None of the C/EBP isoforms tested enhanced the response to PEA3 (data not shown). However, LIP, which functions as a dominant negative C/EBP due to the absence of a transactivation domain (47), caused dose-dependent inhibition of the PEA3 response (Fig. 10*B*). This inhibitory effect of LIP suggests that C/EBP factors may be involved in mediating *COX-2* promoter responsiveness to PEA3 (see "Discussion").

DISCUSSION

Transcriptional up-regulation of *COX-2* has previously been observed under conditions of nuclear β-catenin accumulation, such as in intestinal tumors and *Wnt1*-expressing cell lines. The ability of β-catenin to activate transcription in complex with other transcription factors has led to speculation that β-catenin might directly regulate the *COX-2* promoter. As an initial test of this hypothesis, we examined the regulation of *COX-2* promoter activity by overexpressed β-catenin. We also tested the potential role of PEA3 factors, since we had previously observed coordinate regulation by β-catenin and PEA3 of the matrilysin promoter (42). We observed that β-catenin caused only very weak activation of a *COX-2* promoter reporter construct (Figs. 1*A* and 6). Although it remains possible that

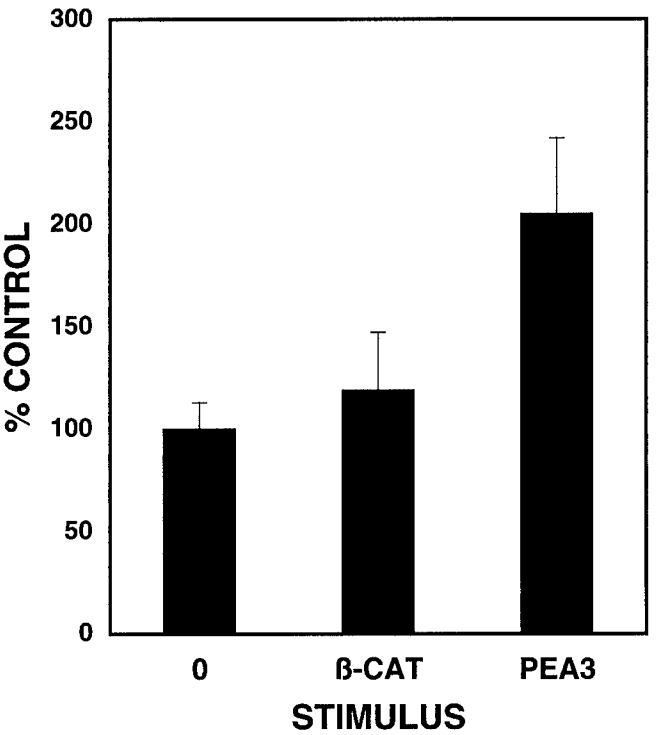

FIG. 6. **PEA3 stimulates *COX-2* promoter activity in C57MG cells.** C57MG cells were transfected with expression vectors encoding β-catenin or PEA3, together with a *COX-2* promoter luciferase reporter construct (COX-2-LUC), and with pRL-TK as an internal control. Luciferase activities were performed as described above. Results shown are the mean + S.D. of five replicates.

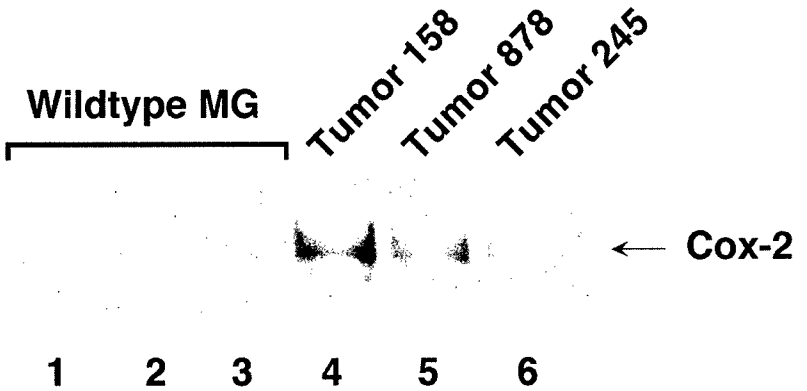

FIG. 7. **Cox-2 protein is increased in *Wnt1*-expressing mammary tumors.** Cox-2 protein was analyzed in lysates prepared from mammary tumors from three *Wnt1* transgenic female mice (*lanes 4–6*) and from mammary glands isolated from three strain-matched wild type female mice (*lanes 1–3*). Lysates were prepared from 10 mg of each tissue sample. Cox-2 protein was immunoprecipitated, and immunoprecipitates were analyzed for Cox-2 by Western blotting as described under "Experimental Procedures." The position of a Cox-2 standard is indicated by the *arrow*. Very little Cox-2 protein was detectable in the wild type mammary glands (*lanes 1–3*). In contrast, significant Cox-2 protein was observed in all three tumor samples (*lanes 4–6*).

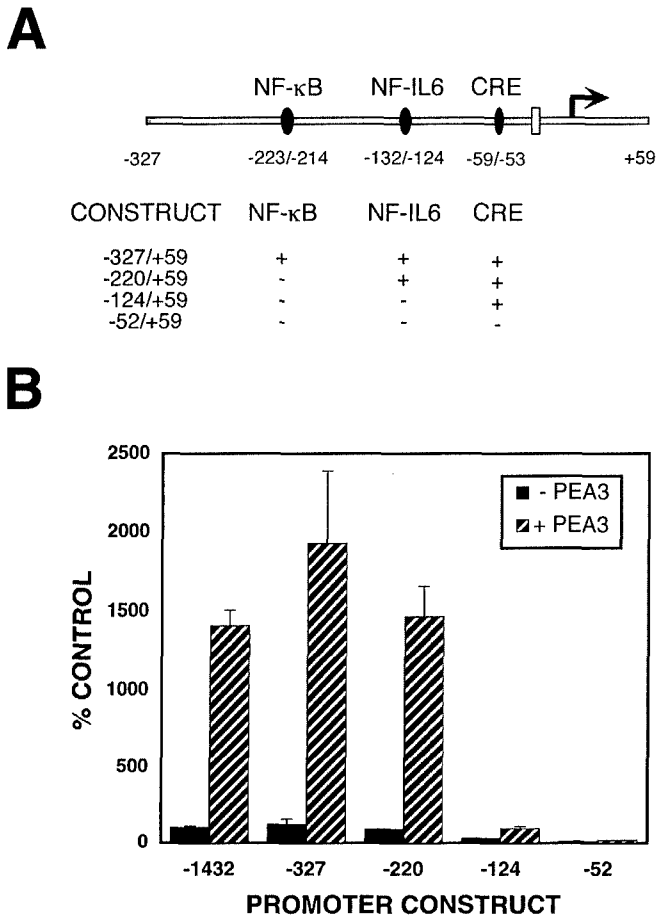

FIG. 8. **Mapping the site of PEA3 responsiveness in the *COX-2* promoter.** *A*, human *COX-2* promoter and deletion constructs. The transcription start site is indicated by an *arrow*, the TATA box at −31/−25 is shown as a *white rectangle*, and three well characterized transcription factor binding sites lying between −327/+59 of the human *COX-2* promoter are depicted as *black ovals*. The presence of these three sites in the truncated constructs used in the experiment shown in *B* are also shown. *B*, promoter truncation analysis. 293 cells were transfected with COX-2-LUC (−1432/+59) or with the truncated *COX-2* promoter constructs shown in *A*, plus pRL-TK, and with or without PEA3 expression vector. Luciferase assays were performed as described above. Results shown are the mean + S.D. of six replicates. *Solid bars*, −PEA3; *hatched bars*, +PEA3.

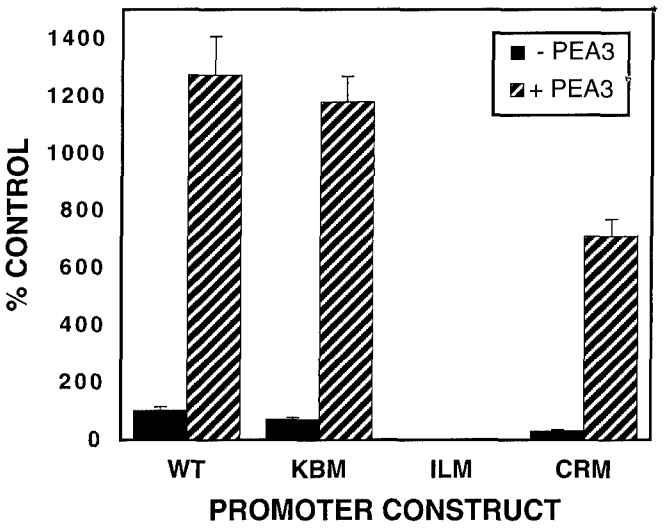

FIG. 9. **Mutation of the NF-IL6-binding site in the *COX-2* promoter destroys PEA3 responsiveness.** 293 cells were transfected with promoter constructs containing residues −327 to +59 of the human *COX-2* promoter, plus pRL-TK, with and without PEA3 expression vector. *WT* is the wild type promoter sequence, KBM has the NF-κB site mutated, ILM has the NF-IL6 site mutated, and CRM has the CRE mutated. Luciferase assays were performed as described above. Results shown are the mean + S.D. of six replicates. *Solid bars*, −PEA3; *hatched bars*, +PEA3.

there are β-catenin-responsive elements lying upstream of the 1.4-kilobase pair promoter sequence used, our data are consistent with the recent report by Haertel-Wiesmann *et al.* (52), who found Wnt-3A-mediated *Cox-2* induction to be resistant to antisense β-catenin oligonucleotides. Furthermore, expression of β-catenin in 293 cells was insufficient to activate transcription from the endogenous *COX-2* gene (Fig. 4). These data suggest that *COX-2* is not a direct target of β-catenin, and raise the possibility that β-catenin may activate *COX-2* via up-regulation of an intermediary transcription factor.

In contrast to the weak response to β-catenin, PEA3 and the related factors ER81 and ERM potently activated the *COX-2* promoter (Figs. 1 and 3). The degree of activation was such that activation of the endogenous *COX-2* gene could be achieved simply by *PEA3* overexpression (Fig. 4). To our knowledge, this is the first report of *COX-2* promoter activation in response to any Ets family transcription factor. Previously, multiple transcription factor binding motifs have been implicated in regulation of the *COX-2* promoter. Thus, both NF-IL6 and CRE sites are important for *Cox-2* induction in mouse mast cells, murine macrophages, and bovine endothelial cells in response to IgE receptor aggregation, lipopolysaccharide, and phorbol ester/lipopolysaccharide, respectively (43, 53, 54). In contrast, tumor necrosis factor-α stimulation of a mouse osteoblastic cell line activates *Cox-2* via the NF-IL6 and NF-κB sites (55), while the CRE site appears critical for *Cox-2* transcription in response to v-Src, platelet-derived growth factor, serum, and ceramide (49, 56, 57). COX-2 can also be regulated post-transcriptionally by mRNA stabilization (58–60).

In this study, we found the NF-IL6 site to be essential for PEA3-mediated *COX-2* promoter induction (Figs. 8 and 9). C/EBPα and C/EBPδ, but not C/EBPβ, caused modest stimulation of the *COX-2* promoter (Fig. 10*A*), as has previously been reported for other cell types (43, 51), but overexpression of wild type C/EBPs did not enhance PEA3 responsiveness. In contrast, a dominant negative C/EBP diminished both basal and PEA3-stimulated promoter activity (Fig. 10*B*). Several models can be proposed to explain these data. PEA3 might bind directly to the NF-IL6 site, in which case the truncated dominant negative C/EBP protein could be antagonistic via competition with PEA3 for the NF-IL6 site. However, the NF-IL6 site does not resemble a consensus PEA3 site (5′-CCGGA(A/T)GC-3′) (61). Alternatively, simultaneous binding of a C/EBP factor to the NF-IL6 site and of PEA3 to a cognate binding site might be required for activation. Several potential Ets-binding sites are present in the minimal PEA3-responsive fragment of the *COX-2* promoter (Fig. 11), although none corresponds exactly to a consensus PEA3 site. Precedents for synergistic transcriptional activation by Ets factors and C/EBP factors have previously been described (62, 63). Thus it seems likely that binding of C/EBP and PEA3 to proximal sites may be required for induction of *COX-2* transcription in our system. However, we have not excluded the possibility that PEA3 might bind to C/EBP proteins at the NF-IL6 site without itself binding to DNA.

As discussed above, NF-IL6 sites in the *COX-2* promoter have previously been implicated in the response to multiple agents (43, 53–55). Interestingly, NF-IL6 sites are also implicated in *COX-2* up-regulation in mouse skin tumors (51), and mutagenesis of the NF-IL6 site reduces *COX-2* promoter activity in the human colon cancer cell lines HCA7 and LS-174 (64).

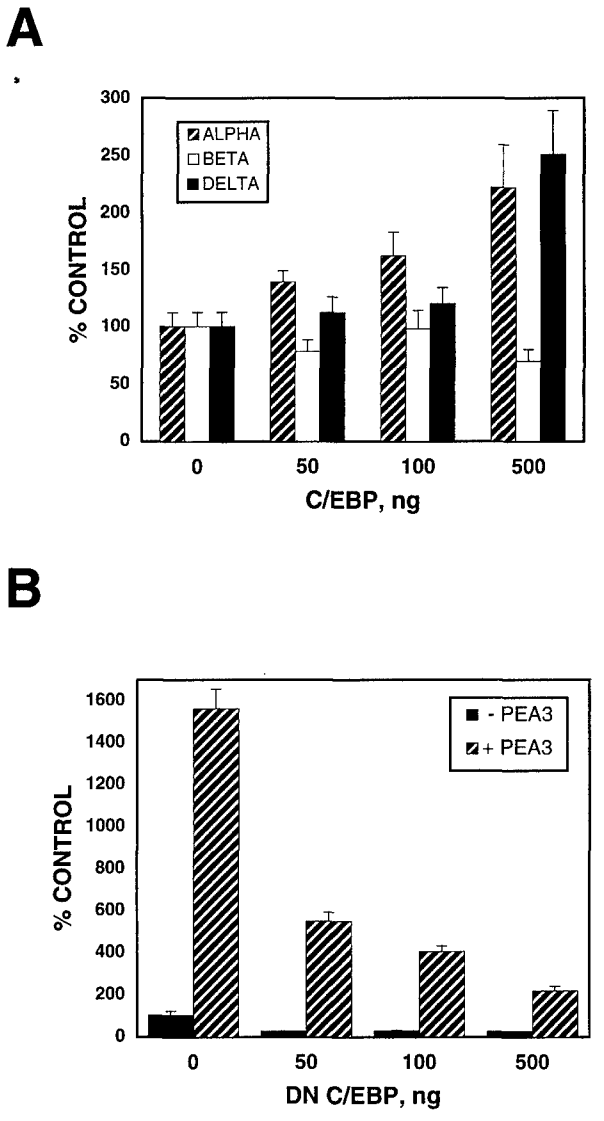

FIG. 10. **Effect of C/EBPs on *COX-2* promoter activity.** *A*, C/EBPα and C/EBPδ, but not C/EBPβ, activate the *COX-2* promoter. 293 cells were transfected with increasing amounts of expression vectors encoding C/EBPα (*hatched bars*), C/EBPβ (*open bars*), or C/EBPδ (*solid bars*), plus COX-2-LUC, and pRL-TK. Luciferase assays were performed as described above. Results shown are the mean + S.D. of six replicates. *B*, dominant negative C/EBP abrogates PEA3-mediated activation of the *COX-2* promoter. 293 cells were transfected with increasing amounts of dominant negative C/EBP expression vector (LIP), plus COX-2-LUC, and pRL-TK with or without PEA3 expression vector. Luciferase assays were performed as described above. Results shown are the mean + S.D. of six replicates. *Solid bars*, −PEA3; *hatched bars*, +PEA3.

This latter result is particularly intriguing since we have previously detected high level *PEA3* expression in intestinal tumors and colorectal cancer-derived cell lines (42), and have also observed PEA3-induced *COX-2* promoter activation in colorectal cancer cell lines.[2] Thus PEA3-mediated regulation of the *COX-2* promoter via the NF-IL6 element may explain the importance of this site in human colorectal cancer lines (64). The correlation between *PEA3* expression and *Cox-2* expression both in intestinal tumors and in *Wnt1*-expressing cells and tumors (Figs. 5 and 7) suggests that PEA3 contributes to *COX-2* up-regulation in response to both *Wnt1* expression and *APC* mutation.

The coincidence of *PEA3* expression in intestinal tumors resulting from *APC* mutation and in mammary tumors caused by *Wnt1* expression suggests that PEA3 up-regulation is a common consequence of activation of the Wnt/β-catenin signaling pathway. This raises the intriguing possibility that PEA3 itself may be a target of β-catenin. To address this directly, the effect of β-catenin on a *PEA3* promoter reporter construct has been assayed. Preliminary data indicate that β-catenin overexpression in Cos cells can stimulate *PEA3* promoter activity up to 5-fold.[3] If PEA3 transcription can be regulated by β-catenin, it is unclear why ectopic expression of β-catenin was insufficient to induce *COX-2* transcription in 293 cells, when PEA3 was sufficient (Figs. 1 and 4). One potential explanation could be differing PEA3 expression levels achieved by transfection with β-catenin and PEA3. Since PEA3 is known to positively regulate its own transcription (65), ectopic PEA3 expression may lead to much greater levels of PEA3 expression than can be achieved in the same time frame by expression of β-catenin. Interestingly, the PEA3 promoters of human, mouse, and chicken all contain a TCF-binding site, conserved in both sequence and position relative to the transcriptional start site,[3] suggesting that PEA3 is most likely a direct target of β-catenin.

PEA3 family members have recently been shown to regulate the promoter of another gene, matrilysin, in synergy with β-catenin (42). Aberrant matrilysin expression is detected in intestinal tumors, and matrilysin is also up-regulated in *Wnt1*-expressing mammary cell lines and tumors.[4] Thus both *COX-2* and *matrilysin* are responsive to Wnt signaling and are regulatable by PEA3 factors. In addition, we have recently observed synergistic activation by β-catenin and PEA3 of the murine *Twist* gene.[5] Finally, we note that the promoters of several genes known to be β-catenin-responsive also contain consensus

[2] H. C. Crawford and L. R. Howe, unpublished data.
[3] C. Messier and J. A. Hassell, unpublished data.
[4] L. R. Howe, O. Watanabe, J. Leonard, and A. M. C. Brown, unpublished data.
[5] L. R. Howe and H. C. Crawford, manuscript in preparation.

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ACTACCCCCTCTGCTCCCAAATTGGGGCAGC**TTCC**TGGGT**TTCC**GATTTTC

TCAT**TTCC**GTGGGTAAAAAACCCTGCCCCCACCGGGCTTACGCAATTTTTT

TAAGGGGAGAGGAG**GGAA**AAATTTGTGGGGGGTACGAAAAGGC**GGAA**AG

AAACAGTCATTTCGTCACATGGGCTTGGTTTTCAGTCTTATAAAAA**GGAA**G

GTTCTCTCGGTTAGCGACCAATTGTCATACGACTTGCAGTGAGCGTCAGG

AGCACGTCCA**GGAA**CTCCTCAGCAGCG

+59

FIG. 11. **Potential Ets sites in the *COX-2* promoter construct −220/+59.** Shown here is the sequence of the human *COX-2* promoter between residues −220 and +59. This is the minimal sequence we have defined which retains PEA3 responsiveness. Thus the sites which confer PEA3 responsiveness must lie in this region. Seven potential Ets-binding sites, defined by the presence of the consensus core motif 5′-GGA(A/T)-3′, are marked in *bold*. In addition, the NF-IL6 site is *underlined*. This site is mutated from 5′-TTACGCAAT-3′ to 5′-TTGGTACCT-3′ in the construct ILM (43).

Ets-binding sites. For example, the *Drosophila* gene *Even-skipped* is coordinately regulated via TCF and Ets-binding sites (66), and the *cyclin D1* promoter can be activated cooperatively by PEA3, β-catenin, and c-Jun.[6] Together these data lead us to speculate that PEA3 factors may contribute to regulation of several target genes of the Wnt/β-catenin pathway.

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REFERENCES

1. Tsukamoto, A. S., Grosschedl, R., Guzman, R. C., Parslow, T., and Varmus, H. E. (1988) *Cell* **55,** 619–625
2. Dale, T. C. (1998) *Biochem. J.* **329,** 209–223
3. Giarre, M., Semenov, M. V., and Brown, A. M. C. (1998) *Ann. N. Y. Acad. Sci.* **857,** 43–55
4. Xu, L., Corcoran, R. B., Welsh, J. W., Pennica, D., and Levine, A. J. (2000) *Genes Dev.* **14,** 585–595
5. Gallet, A., Erkner, A., Charroux, B., Fasano, L., and Kerridge, S. (1998) *Curr. Biol.* **8,** 893–902
6. Zorn, A. M., Barish, G. D., Williams, B. O., Lavender, P., Klymkowsky, M. W., and Varmus, H. E. (1999) *Mol. Cell* **4,** 487–498
7. Polakis, P. (2000) *Genes Dev.* **14,** 1837–1851
8. Crawford, H. C., Fingleton, B. M., Rudolph-Owen, L. A., Goss, K. J., Rubinfeld, B., Polakis, P., and Matrisian, L. M. (1999) *Oncogene* **18,** 2883–2891
9. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998) *Science* **281,** 1509–1512
10. He, T. C., Chan, T. A., Vogelstein, B., and Kinzler, K. W. (1999) *Cell* **99,** 335–345
11. Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., and Ben-Ze'ev, A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 5522–5527
12. Tetsu, O., and McCormick, F. (1999) *Nature* **398,** 422–426
13. Howe, L. R., Subbaramaiah, K., Chung, W. J., Dannenberg, A. J., and Brown, A. M. C. (1999) *Cancer Res.* **59,** 1572–1577
14. Boolbol, S. K., Dannenberg, A. J., Chadburn, A., Martucci, C., Guo, X. J., Ramonetti, J. T., Abreu-Goris, M., Newmark, H. L., Lipkin, M. L., DeCosse, J. J., and Bertagnolli, M. M. (1996) *Cancer Res.* **56,** 2556–2560
15. Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S., and DuBois, R. N. (1994) *Gastroenterology* **107,** 1183–1188
16. Kargman, S. L., O'Neill, G. P., Vickers, P. J., Evans, J. F., Mancini, J. A., and Jothy, S. (1995) *Cancer Res.* **55,** 2556–2559
17. Sano, H., Kawahito, Y., Wilder, R. L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M., and Hla, T. (1995) *Cancer Res.* **55,** 3785–3789
18. Williams, C. S., Luongo, C., Radhika, A., Zhang, T., Lamps, L. W., Nanney, L. B., Beauchamp, R. D., and DuBois, R. N. (1996) *Gastroenterology* **111,** 1134–1140
19. Parrett, M. L., Harris, R. E., Joarder, F. S., Ross, M. S., Clausen, K. P., and Robertson, F. M. (1997) *Int. J. Oncol.* **10,** 503–507
20. Ristimaki, A., Honkanen, N., Jankala, H., Sipponen, P., and Harkonen, M. (1997) *Cancer Res.* **57,** 1276–1280
21. Buckman, S. Y., Gresham, A., Hale, P., Hruza, G., Anast, J., Masferrer, J., and Pentland, A. P. (1998) *Carcinogenesis* **19,** 723–729
22. Chan, G., Boyle, J. O., Yang, E. K., Zhang, F., Sacks, P. G., Shah, J. P., Edelstein, D., Soslow, R. A., Koki, A. T., Woerner, B. M., Masferrer, J. L., and Dannenberg, A. J. (1999) *Cancer Res.* **59,** 991–994
23. Hida, T., Yatabe, Y., Achiwa, H., Muramatsu, H., Kozaki, K., Nakamura, S., Ogawa, M., Mitsudomi, T., Sugiura, T., and Takahashi, T. (1998) *Cancer Res.* **58,** 3761–3764
24. Wolff, H., Saukkonen, K., Anttila, S., Karjalainen, A., Vainio, H., and Ristimaki, A. (1998) *Cancer Res.* **58,** 4997–5001
25. Liu, C. H., Chang, S.-H., Narko, K., Trifan, O. C., Wu, M.-T., Smith, E., Haudenschild, C., Lane, T. F., and Hla, T. (2001) *J. Biol. Chem.* **276,** in press
26. Fischer, S. M., Lo, H. H., Gordon, G. B., Seibert, K., Kelloff, G., Lubet, R. A., and Conti, C. J. (1999) *Mol. Carcinog.* **25,** 231–240
27. Harris, R. E., Alshafie, G. A., Abou-Issa, H., and Seibert, K. (2000) *Cancer Res.* **60,** 2101–2103
28. Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. (1996) *Cell* **87,** 803–809
29. Reddy, B. S., Hirose, Y., Lubet, R., Steele, V., Kelloff, G., Paulson, S., Seibert, K., and Rao, C. V. (2000) *Cancer Res.* **60,** 293–297
30. Tiano, H., Chulada, P., Spalding, J., Lee, C., Loftin, C., Mahler, J., Morham, S., and Langenbach, R. (1997) *Proc. Am. Assn. Cancer Res.* **38,** 1727
31. Steinbach, G., Lynch, P. M., Phillips, R. K., Wallace, M. H., Hawk, E., Gordon, G. B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L. K., and Levin, B. (2000) *N. Engl. J. Med.* **342,** 1946–1952
32. Tsujii, M., and DuBois, R. N. (1995) *Cell* **83,** 493–501
33. Tsujii, M., Kawano, S., and DuBois, R. N. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3336–3340
34. Masferrer, J. L., Leahy, K. M., Koki, A. T., Zweifel, B. S., Settle, S. L., Woerner, B. M., Edwards, D. A., Flickinger, A. G., Moore, R. J., and Seibert, K. (2000) *Cancer Res.* **60,** 1306–1311
35. Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M., and DuBois, R. N. (1998) *Cell* **93,** 705–716
36. Williams, C. S., Tsujii, M., Reese, J., Dey, S. K., and DuBois, R. N. (2000) *J. Clin. Invest.* **105,** 1589–1594
37. Yamada, M., Kawai, M., Kawai, Y., and Mashima, Y. (1999) *Curr. Eye Res.* **19,** 300–304
38. Majima, M., Isono, M., Ikeda, Y., Hayashi, I., Hatanaka, K., Harada, Y., Katsumata, O., Yamashina, S., Katori, M., and Yamamoto, S. (1997) *Jpn J. Pharmacol.* **75,** 105–114
39. Seed, M. P., Brown, J. R., Freemantle, C. N., Papworth, J. L., Colville-Nash, P. R., Willis, D., Somerville, K. W., Asculai, S., and Willoughby, D. A. (1997) *Cancer Res.* **57,** 1625–1629
40. Howe, L. R., Leevers, S. J., Gomez, N., Nakielny, S., Cohen, P., and Marshall, C. J. (1992) *Cell* **71,** 335–342
41. Xin, J. H., Cowie, A., Lachance, P., and Hassell, J. A. (1992) *Genes Dev.* **6,** 481–496
42. Crawford, H. C., Fingleton, B., Gustavson, M. D., Kurpios, N., Wagenaar, R. A., Hassell, J. A., and Matrisian, L. M. (2001) *Mol. Cell. Biol.* **21,** 1370–1383
43. Inoue, H., Yokoyama, C., Hara, S., Tone, Y., and Tanabe, T. (1995) *J. Biol. Chem.* **270,** 24965–24971
44. van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., and Clevers, H. (1997) *Cell* **88,** 789–799
45. Kypta, R. M., Su, H., and Reichardt, L. F. (1996) *J. Cell Biol.* **134,** 1519–1529
46. Descombes, P., Chojkier, M., Lichtsteiner, S., Falvey, E., and Schibler, U. (1990) *Genes Dev.* **4,** 1541–1551
47. Descombes, P., and Schibler, U. (1991) *Cell* **67,** 569–579
48. Brown, A. M. C., Wildin, R. S., Prendergast, T. J., and Varmus, H. E. (1986) *Cell* **46,** 1001–1009
49. Subbaramaiah, K., Chung, W. J., and Dannenberg, A. J. (1998) *J. Biol. Chem.* **273,** 32943–32949
50. Lekstrom-Himes, J., and Xanthopoulos, K. G. (1998) *J. Biol. Chem.* **273,** 28545–28548
51. Kim, Y., and Fischer, S. M. (1998) *J. Biol. Chem.* **273,** 27686–27694
52. Haertel-Wiesmann, M., Liang, Y., Fantl, W. J., and Williams, L. T. (2000) *J. Biol. Chem.* **275,** 32046–32051
53. Reddy, S. T., Wadleigh, D. J., and Herschman, H. R. (2000) *J. Biol. Chem.* **275,** 3107–3113
54. Wadleigh, D. J., Reddy, S. T., Kopp, E., Ghosh, S., and Herschman, H. R. (2000) *J. Biol. Chem.* **275,** 6259–6266
55. Yamamoto, K., Arakawa, T., Ueda, N., and Yamamoto, S. (1995) *J. Biol. Chem.* **270,** 31315–31320
56. Xie, W., and Herschman, H. R. (1995) *J. Biol. Chem.* **270,** 27622–27628
57. Xie, W., and Herschman, H. R. (1996) *J. Biol. Chem.* **271,** 31742–31748
58. Ristimaki, A., Garfinkel, S., Wessendorf, J., Maciag, T., and Hla, T. (1994) *J. Biol. Chem.* **269,** 11769–11775
59. Ristimaki, A., Narko, K., and Hla, T. (1996) *Biochem. J.* **318,** 325–331
60. Sheng, H., Williams, C. S., Shao, J., Liang, P., DuBois, R. N., and Beauchamp, R. D. (1998) *J. Biol. Chem.* **273,** 22120–22177
61. Brown, L. A., Amores, A., Schilling, T. F., Jowett, T., Baert, J. L., de Launoit, Y., and Sharrocks, A. D. (1998) *Oncogene* **17,** 93–104
62. McNagny, K. M., Sieweke, M. H., Doderlein, G., Graf, T., and Nerlov, C. (1998) *EMBO J.* **17,** 3669–3680
63. Nagulapalli, S., Pongubala, J. M., and Atchison, M. L. (1995) *J. Immunol.* **155,** 4330–4338
64. Shao, J., Sheng, H., Inoue, H., Morrow, J. D., and DuBois, R. N. (2000) *J. Biol. Chem.* **275,** 33951–33956
65. Benz, C. C., O'Hagan, R. C., Richter, B., Scott, G. K., Chang, C. H., Xiong, X., Chew, K., Ljung, B. M., Edgerton, S., Thor, A., and Hassell, J. A. (1997) *Oncogene* **15,** 1513–1525
66. Halfon, M. S., Carmena, A., Gisselbrecht, S., Sackerson, C. M., Jimenez, F., Baylies, M. K., and Michelson, A. M. (2000) *Cell* **103,** 63–74

[6] C. Messier, H. C. Crawford, and J. A. Hassell, unpublished data.

Endocrine-Related Cancer (2001) **8** 97–114

Cyclooxygenase-2: a target for the prevention and treatment of breast cancer

L R Howe[1,2], *K Subbaramaiah*[1,3], *A M C Brown*[1,2] *and A J Dannenberg*[1,3]

[1]Strang Cancer Research Laboratory, Rockefeller University, Box 231, 1230 York Avenue, New York, New York 10021, USA
[2]Department of Cell Biology and Anatomy, Weill Medical College of Cornell University, New York, New York 10021, USA
[3]Department of Medicine, Weill Medical College of Cornell University, New York, New York 10021, USA

(Requests for offprints should be addressed to L R Howe, Strang Cancer Research Laboratory, Rockefeller University, Box 231, 1230 York Avenue, New York, New York 10021, USA; Email: lrhowe@med.cornell.edu)

Abstract

Cyclooxygenase-2 (COX-2), an inducible prostaglandin synthase, is normally expressed in parts of the kidney and brain. Aberrant *COX-2* expression was first reported in colorectal carcinomas and adenomas, and has now been detected in various human cancers, including those of the breast. Strikingly, *COX-2* overexpression in murine mammary gland is sufficient to cause tumour formation. To date, the role of COX-2 in tumorigenesis has been most intensively studied in the colon. Thus, the relationship between COX-2 and neoplasia can best be illustrated with reference to intestinal tumorigenesis. Here we consider the potential utility of selective COX-2 inhibitors for the prevention and treatment of breast cancer. Data for cancers of the colon and breast are compared where possible. In addition, the mechanisms by which *COX-2* is upregulated in cancers and contributes to tumorigenesis are discussed. Importantly, several recent studies of mammary tumorigenesis in animal models have found selective COX-2 inhibitors to be effective in the prevention and treatment of breast cancer. Clinical trials will be needed to determine whether COX-2 inhibition represents a useful approach to preventing or treating human breast cancer.

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Introduction

Cyclooxygenase-2 (COX-2) is emerging as an increasingly promising pharmacological target for the prevention and treatment of many human cancers. COX-1 and COX-2 are prostaglandin (PG) synthases which catalyse sequential synthesis of prostaglandin G_2 (PGG_2) and PGH_2 from arachidonic acid by virtue of intrinsic cyclooxygenase and peroxidase activities (Fig. 1). PGH_2 is then converted by specific isomerases to other eicosanoids, including PGs, thromboxane (Tx) and prostacyclin. Cyclooxygenase-derived prostanoids contribute to many normal physiological processes including haemostasis, platelet aggregation, kidney and gastric function, reproduction, pain and fever. Despite the similar enzymatic activities of COX-1 and COX-2, the *COX-1* and *COX-2* genes have distinct properties, and differing expression patterns (Table 1). While *COX-1* is constitutively expressed, *COX-2* is upregulated in response to growth factors, tumour promoters and cytokines (reviewed by Herschman 1996). Additionally, *COX-2* is responsive to several oncogenes, including v-*src*, v-*Ha-ras*, *HER-2/neu* and *Wnt* genes (Xie & Herschman 1995, Subbaramaiah *et al.* 1996, Sheng *et al.* 1998*b*, Howe *et al.* 1999, Vadlamudi *et al.* 1999, Haertel-Wiesmann *et al.* 2000). Thus, increased PG synthesis is detected in inflamed and neoplastic tissues. Analysis of *COX-2*-deficient mice suggests that COX-2 is normally important for post-natal renal development and multiple female reproductive processes including ovulation, fertilisation, implantation and decidualisation (Dinchuk *et al.* 1995, Morham *et al.* 1995, Lim *et al.* 1997, 1999*a*). Aberrant *COX-2* expression has been detected in multiple human cancers, as shown in Table 2. Together, a weight of epidemiological, pharmacological, genetic and expression data combine to suggest an important role for COX-2 in tumorigenesis, particularly in colorectal cancer. There is recent evidence that COX-2 may also represent a novel target for the prevention and treatment of breast cancer.

Cyclooxygenase activity is inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and sulindac, which are most commonly administered for the relief of pain and inflammation. However, adverse side effects including peptic ulcer disease are associated with the use of such compounds, which are nonselective inhibitors of

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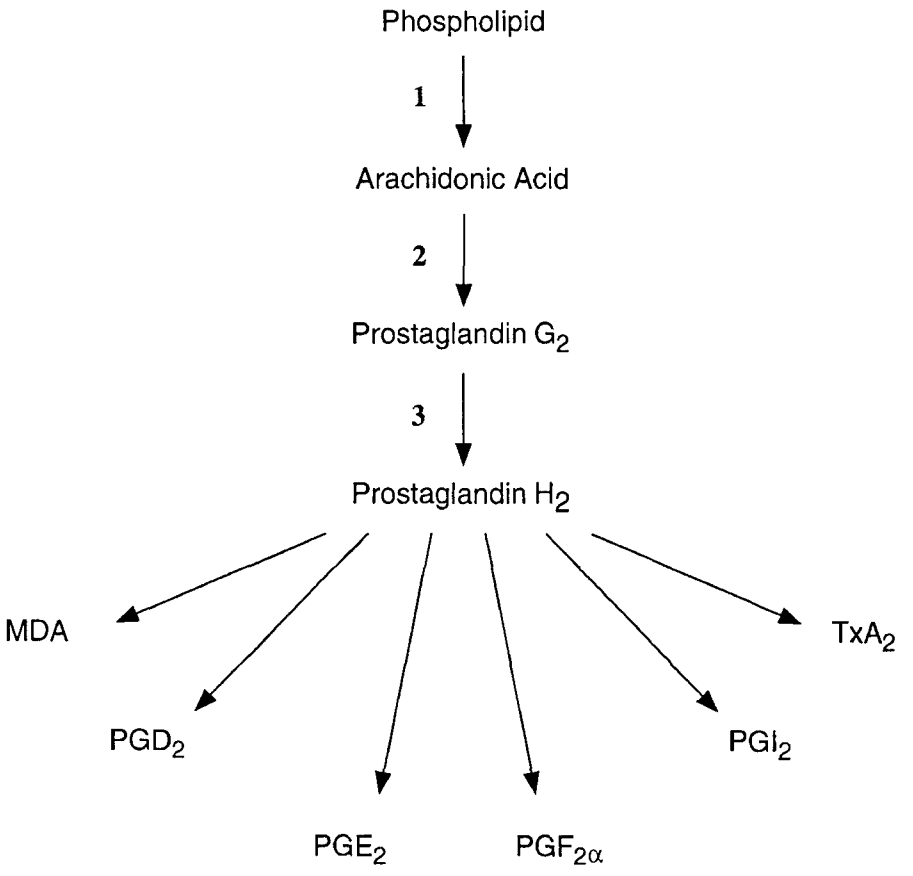

Figure 1 Biosynthesis of prostaglandins. Arachidonic acid, released from membrane phospholipids by phospholipase A_2 action (reaction 1), is metabolised by cyclooxygenases to PGH_2 in two steps. PGG_2 is generated by cyclooxygenase activity (reaction 2), then converted to PGH_2 by the peroxidase activity (reaction 3); both enzyme activities are intrinsic to COX-1 and COX-2. PGH_2 can be converted to several eicosanoids by specific isomerases. Additionally, MDA (malondialdehyde) can be produced enzymatically or by degradation of PGH_2. TxA_2, thromboxane A_2.

Table 1 Properties of COX-1 and COX-2.

	COX-1	COX-2
Expression	Constitutive	Inducible
Size of gene	22 kb	8.3 kb
mRNA transcript	2.7 kb	4.5 kb, with multiple Shaw-Kamen sequences
Size of protein	72 kDa	72/74 kDa doublet
Localisation	Endoplasmic reticulum, nuclear envelope	Endoplasmic reticulum, nuclear envelope
Expression pattern	Most tissues, including stomach, kidney, colon and platelets	Regions of brain and kidney, activated macrophages, synoviocytes during inflammation, malignant epithelial cells. Expression stimulated by cytokines, growth factors, oncogenes and tumour promoters

COX-1 and COX-2. In fact, prior to the development of selective COX-2 inhibitors, there were an estimated 100 000 hospitalisations and 16 500 deaths per year in the United States related to NSAID use (Singh 1998). Toxicity associated with the use of nonselective NSAIDs was the major stimulus to develop selective COX-2 inhibitors. Endoscopically controlled studies show that selective COX-2 inhibitors are far less ulcerogenic than classical NSAIDs (Langman *et al.* 1999, Simon *et al.* 1999). Since selective COX-2 inhibitors appear sufficiently safe to allow large scale administration on a chronic basis to healthy individuals, they represent potentially useful agents for cancer chemoprevention.

COX-2 and cancer: epidemiology and expression

Colon cancer

One of the first clues that cyclooxygenase inhibition might be an effective approach to preventing cancer came from

Table 2 *COX-2* overexpression in human tumours.

Organ site	References
Breast cancer	Parrett *et al.* (1997), Hwang *et al.* (1998), Masferrer *et al.* (2000), Subbaramaiah *et al.* (1999*b*), Soslow *et al.* (2000)
Cervical dysplasia and cancer	Kulkarni *et al.* (2001)
Prostate carcinoma	Gupta *et al.* (2000), Yoshimura *et al.* (2000)
Bladder transitional cell carcinoma	Mohammed *et al.* (1999)
Hepatocellular carcinoma	Koga *et al.* (1999)
Pancreatic cancer	Molina *et al.* (1999), Okami *et al.* (1999), Tucker *et al.* (1999)
Skin cancer	Buckman *et al.* (1998)
Lung cancer	Hida *et al.* (1998), Wolff *et al.* (1998) Ochiai *et al.* (1999)
Head and neck cancer	Chan *et al.* (1999)
Colorectal adenomas and carcinomas	Eberhart *et al.* (1994), Kargman *et al.* (1995), Sano *et al.* (1995), Kutchera *et al.* (1996)
Gastric cancer	Ristimaki *et al.* (1997)
Barrett's oesophagus and oesophageal cancer	Wilson *et al.* (1998)

epidemiological studies. Several studies reported an inverse correlation between colon cancer incidence and regular use of NSAIDs including aspirin (Thun *et al.* 1991, Greenberg *et al.* 1993, Logan *et al.* 1993, Suh *et al.* 1993, Reeves *et al.* 1996). Since NSAIDs are known to function, at least in part, by inhibiting cyclooxygenase enzyme activity, these observations suggested that aberrant PG biosynthesis might contribute to colorectal neoplasia. This led to an analysis of *COX* expression in colorectal neoplasms. Levels of *COX-1* were not increased in colorectal carcinomas relative to adjacent normal mucosa (Eberhart *et al.* 1994, Kargman *et al.* 1995, Sano *et al.* 1995). In contrast, striking *COX-2* upregulation was observed in colon carcinomas compared with the virtually undetectable expression in normal mucosa (Eberhart *et al.* 1994, Kargman *et al.* 1995, Sano *et al.* 1995, Kutchera *et al.* 1996). Eberhart *et al.* (1994) also detected *COX-2* expression in 9 of 20 adenomas examined. In carcinomas, COX-2 protein localised predominantly to the epithelial component, but could also be detected in tumour-associated fibroblasts, vascular endothelial cells, and inflammatory mononuclear cells (Sano *et al.* 1995, Kutchera *et al.* 1996). *COX-2* expression has also been detected in intestinal adenomas from rodent models of intestinal tumorigenesis (Boolbol *et al.* 1996, DuBois *et al.* 1996*a*, Williams *et al.* 1996, Singh *et al.* 1997).

Together, these epidemiological and expression studies suggested a role for COX-2 in colorectal tumorigenesis. This idea is supported by the results of clinical trials. Treatment with the NSAID sulindac or with celecoxib, a selective COX-2 inhibitor, causes a decrease in the size and number of polyps in familial adenomatous polyposis patients (Giardiello *et al.* 1993, Steinbach *et al.* 2000). Thus, overexpression of *COX-2* appears to contribute to colorectal cancer and cyclooxygenase inhibitors are likely to be useful chemopreventive agents.

Breast cancer

In contrast to colon cancer, the role of COX-2 in breast cancer is less clear. Epidemiological studies conducted to investigate the relationship between NSAID use and breast cancer incidence have reported conflicting findings. Several studies have failed to find a significant relationship between aspirin use and breast cancer risk (Paganini-Hill *et al.* 1989, Thun *et al.* 1991, Egan *et al.* 1996). However, other analyses have revealed an association between NSAID consumption and decreased breast cancer incidence. Friedman & Ury (1980) found significantly reduced breast cancer incidence in 4867 women who used indomethacin, compared with age-matched controls. Harris and colleagues (1996) compared NSAID use in 511 women with newly diagnosed breast cancer with 1534 population control subjects, and found that the relative risk of breast cancer was reduced to 66% in women using NSAIDs at least 3 times per week for at least one year. Two additional studies also found that NSAIDs protected against breast cancer (Schreinemachers & Everson 1994, Sharpe *et al.* 2000). The basis for the lack of consistency among different studies is unclear. One potential explanation is that some NSAIDs may have restricted bioavailability in breast tissue. Thus, conflicting data obtained in separate studies may reflect the usage of different NSAIDs in the populations examined. Another potential complication is that significant *COX-2* overexpression may be limited to a subset of human breast cancers, which could certainly confound epidemiological analyses. Approximately 85% of human colorectal adenocarcinomas overexpress *COX-2* (Eberhart *et al.* 1994, Kargman *et al.* 1995, Sano *et al.* 1995, Kutchera *et al.* 1996). This could account for the strong correlation between regular NSAID use and reduced cancer incidence (Thun *et al.* 1991, Greenberg *et al.* 1993, Logan *et al.* 1993, Suh *et al.* 1993, Reeves *et al.* 1996). In contrast, as discussed below, *COX-2* is not abundantly overexpressed in the majority of human breast cancers (Hwang *et al.* 1998, Subbaramaiah *et al.* 1999*b*). With this in mind, it is predictable that the results of epidemiological studies would be less clear-cut for breast than colon cancer even if NSAIDs were active against COX-2-positive breast cancers.

Enhanced *COX* expression in breast cancer was first suggested by reports of elevated PG levels in breast tumours (Tan *et al.* 1974, Bennett *et al.* 1977, Rolland *et al.* 1980). PG production was increased in human breast cancers, particularly in those from patients with metastatic disease (Bennett *et al.*

1977, Rolland *et al.* 1980). PG production and *COX-2* expression have also been detected in breast cancer-derived cell lines (Schrey & Patel 1995, Liu & Rose 1996, Gilhooly & Rose 1999). Interestingly, there appears to be a correlation between invasiveness/metastatic potential and PG production/*COX-2* expression in both cell lines and tumour specimens (Bennett *et al.* 1977, Rolland *et al.* 1980, Liu & Rose 1996). However, there are conflicting data regarding the frequency of *COX-2* expression in breast cancers. Parrett *et al.* (1997) detected *COX-2* expression in 13/13 human breast tumours by reverse transcriptase-coupled polymerase chain reaction (RT-PCR), compared with no detectable expression in normal human breast tissue, and observed a correlation between *COX-2* expression and increasing tumour cell density. Immunohistochemistry revealed COX-2 protein in the epithelial cells of the tumours, with no expression in the stromal compartment. In contrast, Hwang and colleagues (1998) analysed 44 tumour samples by Western blotting but only detected COX-2 protein in 2 of the 44 samples. These apparently discrepant observations can be reconciled by consideration of the following. First, the failure to detect COX-2 by Western blotting or RNAse protection (Hwang *et al.* 1998) may reflect the relative insensitivity of these techniques compared with RT-PCR. The second important caveat is that *COX-2* expression may be predominantly associated with certain subsets of human breast cancers (Gilhooly & Rose 1999, Subbaramaiah *et al.* 1999*b*). We have examined *COX-2* expression in 29 microdissected human breast cancers using a coupled immunoprecipitation/Western blotting assay, which confers increased sensitivity relative to direct Western blotting of lysates. High levels of COX-2 protein were detected in 14 of 15 *HER-2/neu*-overexpressing breast cancers. In contrast, COX-2 was detected in only 4 of 14 HER-2/neu-negative breast cancers, and was expressed at significantly lower levels than in the HER-2/neu-positive samples (Subbaramaiah *et al.* 1999*b*). Immunohistochemistry localised COX-2 protein to epithelial cells and the vasculature. Thus, it seems likely that significant overexpression of *COX-2* may be largely confined to those breast cancers in which *HER-2/neu* is overexpressed, or in which the signalling pathways normally activated by HER-2/neu are activated by an alternative event such as *ras* mutation (Gilhooly & Rose 1999). Since *HER-2/neu* overexpression is limited to 20–30% of human breast cancers, conflicting epidemiological data may reflect differing proportions of HER-2/neu-positive cancers in the various studies. Based on these recent findings, it would be of considerable interest to compare the efficacy of NSAIDs in preventing HER-2/neu-positive and -negative breast cancers.

COX-2 is expressed in intestinal and mammary tumours in rodents

Rodent models of intestinal tumorigenesis can be divided into carcinogen-induced tumour models, and those in which tumour formation is induced by introduction of germline mutations into tumour suppressor genes such as *Apc*. In humans, germline mutation of the *APC* gene is responsible for familial adenomatous polyposis (FAP), in which individuals develop numerous adenomatous colorectal polyps, which predispose to colorectal carcinomas. In addition, *APC* is mutated in approximately 85% of sporadic colorectal carcinomas. Several mice strains have been developed which harbour mutations in one *Apc* allele, including the Min mouse (Moser *et al.* 1990), $Apc^{\Delta 716}$ (Oshima *et al.* 1995), Apc1638N (Fodde *et al.* 1994), and $Apc^{\Delta 474}$ (Sasai *et al.* 2000). These mice consistently develop intestinal adenomas, although these are more prevalent in the small intestine than in the colon. Analysis of adenomatous polyps from Min mice revealed increased *COX-2* expression relative to normal mucosa (Williams *et al.* 1996). Elevated expression of *COX-2* was also detected in colonic mucosa and tumours from rats treated with azoxymethane (AOM) (DuBois *et al.* 1996*a*, Singh *et al.* 1997). Thus *COX-2* is commonly overexpressed in both human colorectal cancers and animal models of colorectal cancer. The cellular localisation of COX-2 in both human and rodent tumours continues to be investigated.

Rodent models have also been used to examine *COX-2* expression in mammary tumours. In the rat, *COX-1* is ubiquitously expressed in virgin, pregnant, lactating, and post-lactational mammary glands, but *COX-2* is only detectable in the mammary glands of lactating animals (Badawi *et al.* 1999). Treatment of ovariectomised animals with oestradiol and progesterone causes induction of *COX-2* and PG synthesis (Badawi & Archer 1998, Badawi *et al.* 1999), suggesting that *COX-2* transcription is susceptible to hormonal regulation. COX-2 protein has been detected in rat mammary tumours induced by various carcinogens, including *N*-nitrosomethyl urea (NMU), dimethyl-benz[a]anthracene (DMBA) and 2-amino-1-methyl-6-phenylimidazol[4,5-b]pyridine (PhIP) (Robertson *et al.* 1998, Hamid *et al.* 1999, Nakatsugi *et al.* 2000). Based on immunohistochemical analyses, COX-2 protein was observed in the epithelial cells within the mammary tumours (Robertson *et al.* 1998, Nakatsugi *et al.* 2000). Interestingly, dietary administration of n-6 polyunsaturated fatty acids (PUFAs) in the form of safflower oil stimulated *COX-2* expression in rat mammary glands, suggesting a potential mechanism by which n-6-PUFAs may contribute to mammary tumorigenesis (Badawi *et al.* 1998).

In addition to these rat studies, COX-2 protein levels have also been examined in mammary tissues from transgenic mice strains that develop mammary tumours due to mammary-targeted oncogene expression. Significant amounts of COX-2 protein were detected in mammary tumours from mice overexpressing *neu* (K Subbaramaiah and A J Dannenberg, unpublished observations), consistent with our findings in *HER-2/neu*-overexpressing human

breast cancers (Subbaramaiah *et al.* 1999*b*). We have also found increased COX-2 protein in mammary tumours from *Wnt-1* transgenic mice, relative to the levels in normal mammary gland (Fig. 2; Howe *et al.* 2001). Consistent with this, *COX-2* is transcriptionally upregulated in mouse mammary epithelial cell lines engineered to express *Wnt-1* (Howe *et al.* 1999), and expression is also increased in response to transformation by other oncogenes (Subbaramaiah *et al.* 1996).

Mechanisms of *COX-2* upregulation

There is evidence that *COX-2* is upregulated in both neoplastic and stromal cells within tumours. Hence, multiple mechanisms are likely to account for overexpression of *COX-2* in these different cell types. It is relevant, therefore, to evaluate the effects of different stimuli in various cell types. *COX-2* expression is normally regulated at both transcriptional and post-transcriptional levels, and can also be regulated by the rate of protein synthesis and/or degradation. The human *COX-2* promoter contains multiple transcription factor binding sites, including a cAMP response element (CRE), and potential binding sites for Myb, nuclear factor interleukin-6 (NF-IL6), nuclear factor κB (NF-κB), and Ets factors (Genbank Accession Number 505116). Of these, the sites proximal to the transcription start site (Fig. 3) have been shown to be differentially responsive to various stimuli. Induction of *COX-2* by v-*src*, serum, platelet-derived growth factor (PDGF) and ceramide requires activation of both Ras/Raf-1/ERK and Ras/MEKK1/JNK signal transduction pathways and is predominantly mediated via the CRE (Xie & Herschman 1995, 1996, Subbaramaiah *et al.* 1998*a*). In contrast, the NF-IL6 and NF-κB sites are required for induction of *COX-2* in response to tumour necrosis factor (TNF) in osteoblasts (Yamamoto *et al.* 1995). The NF-IL6 and CRE sites have been identified as being critical for the induction of *COX-2* in response to other stimuli, including lipopolysaccharide (LPS) and immunoglobulin E receptor aggregation (Inoue *et al.* 1995, Reddy *et al.* 2000*b*, Wadleigh *et al.* 2000). Other studies have implicated the NF-κB site as being important for LPS- and benzo[a]pyrene-mediated induction of *COX-2* (Hwang *et al.* 1997, Yan *et al.* 2000). The expression of *COX-2* can also be increased by stabilisation of the *COX-2* transcript (Ristimaki *et al.* 1994, Sheng *et al.* 1998*b*). The 3′ untranslated region of *COX-2* mRNA contains a 116 nucleotide AU-rich sequence element (ARE) which can negatively regulate transcript stability and modulate translation (Dixon *et al.* 2000).

During tumorigenesis, increased expression of *COX-2* is likely to be a consequence of multiple effects. For example, transcriptional activation is likely to occur in response to growth factors and oncogenes. Moreover, since wildtype p53 decreases *COX-2* transcription, loss-of-function p53 mutations may contribute to *COX-2* upregulation (Subbaramaiah *et al.* 1999*a*). Dixon *et al.* (2000) speculated that ARE-binding proteins which normally negatively regulate transcript stability may be defective in tumour cells. This, too, could result in increased levels of COX-2. The relative importance of these different factors is likely to vary in different tissues. In mouse skin carcinogenesis, promoter activation by upstream stimulatory factor (USF) and CCAAT/enhancer binding proteins (C/EBPs) appears to be important (Kim & Fischer 1998). By contrast, bile acids, which have been implicated in the promotion of

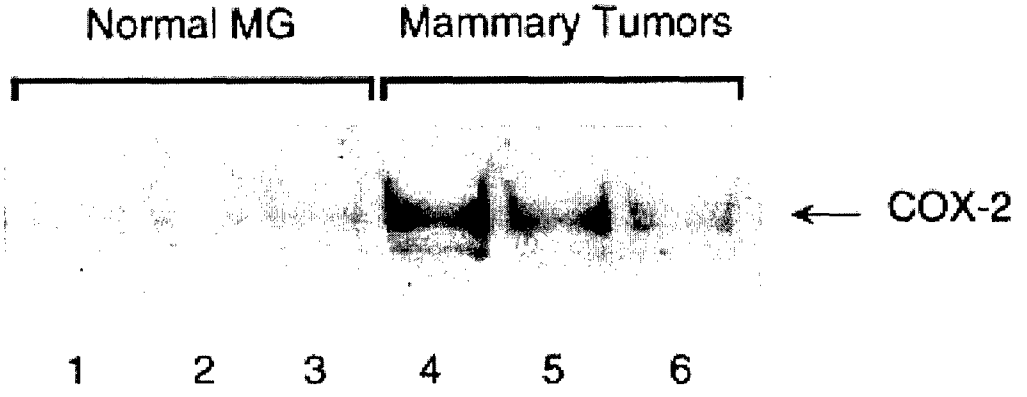

Figure 2 COX-2 protein is increased in *Wnt-1*-expressing mammary tumours. COX-2 protein was analysed in lysates prepared from mammary tumours from three *Wnt-1* transgenic female mice (lanes 4–6) and from mammary glands (MG) isolated from three strain-matched wildtype female mice (lanes 1–3). Lysates were prepared from 10 mg of each tissue sample. COX-2 protein was immunoprecipitated, and immunoprecipitates were analysed for COX-2 by Western blotting. The arrow indicates the position of a COX-2 standard. Little COX-2 protein was detectable in the wildtype mammary glands (lanes 1–3). In contrast, appreciable COX-2 protein was observed in all three tumour samples (lanes 4–6). Adapted and reproduced with permission from Howe *et al.* (2001).

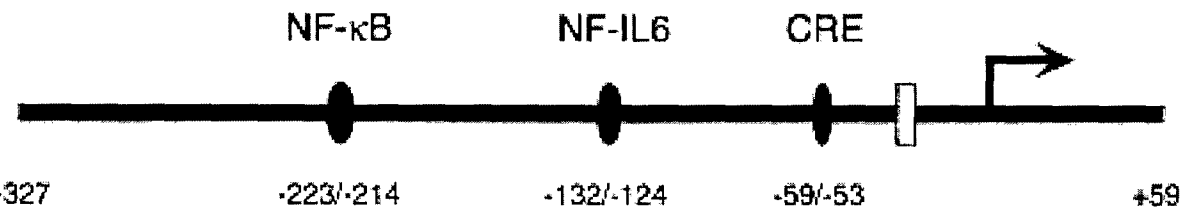

Figure 3 Human *COX-2* promoter schematic. The transcription start site is indicated by an arrow, the TATA box at −31/−25 is shown as a white rectangle, and three transcription factor binding sites lying between −327/+59 of the human *COX-2* promoter are depicted as black ovals.

gastrointestinal tumours, stimulate AP-1 activity and increase *COX-2* transcription and transcript stability (Zhang *et al.* 1998, Zhang *et al.* 2000*b*).

In colorectal cancer cells, constitutive *COX-2* expression is likely to result from a combination of transcriptional, post-transcriptional and translational effects (Hsi *et al.* 1999, Shao *et al.* 2000). Mutation of NF-IL6 and CRE elements has been shown to diminish *COX-2* promoter activity in two colorectal cancer cell lines (Shao *et al.* 2000), implicating these sites in transcriptional upregulation. Interestingly, we have recently detected a requirement for the NF-IL6 site for stimulation of the *COX-2* promoter by Ets factors of the PEA3 subfamily (Howe *et al.* 2001). PEA3 factors stimulate human *COX-2* promoter activity up to 20-fold when overexpressed in 293 human embryonic kidney cells. Since PEA3 factors are highly expressed in colorectal cancer cell lines, intestinal tumours, and *Wnt-1*-expressing mammary cell lines and tumours (Crawford *et al.* 2001, Howe *et al.* 2001), we speculate that PEA3 factors may contribute to *COX-2* induction during both intestinal and mammary tumorigenesis. Increased expression of c-*myb* may also contribute, since c-*myb* is upregulated in colon tumours and breast cancers (Guerin *et al.* 1990, Ramsay *et al.* 1992) and c-*myb* overexpression causes modest induction of *COX-2* promoter activity (Ramsay *et al.* 2000).

COX-2 expression can also be affected by dietary fat. Chemically induced mammary carcinogenesis is promoted by dietary n-6-PUFAs, which enhance tissue levels of arachidonic acid, and inhibited by n-3-PUFAs (reviewed by Rose & Connolly 1999). In experimental models, *COX-2* expression in mammary tissue and tumours is decreased in animals fed an n-3-PUFA-rich diet (i.e. menhaden oil) relative to those fed a diet high in n-6-PUFAs (i.e. corn or safflower oil) (Badawi *et al.* 1998, Hamid *et al.* 1999). Similarly, dietary fish oil decreased the expression of *COX-2* and the incidence of colorectal tumours in AOM-treated rats (Singh *et al.* 1997). This may help to explain epidemiological observations of decreased breast and colon cancer risk in populations with diets rich in fish oils (Rose & Connolly 1999).

Evidence from rodent models that COX-2 contributes to cancer

Genetic evidence for a role for COX-2 in tumour formation

Definitive evidence linking cyclooxygenases to tumorigenesis was first provided by studies using mice with targeted disruptions of the *COX-1* or *COX-2* genes. Oshima *et al.* (1996) pioneered these experiments by generating $Apc^{\Delta 716}$, *COX-2*-null mice. Intestinal adenoma incidence was reduced by 86% in *COX-2* knockout mice, and by 66% in *COX-2* heterozygotes, relative to *COX-2* wildtype mice carrying the *Apc* mutation (Oshima *et al.* 1996). Tumour size was also significantly reduced in *COX-2*-deficient mice. *COX-2* deficiency also protects against chemically induced papilloma formation in mouse skin (Tiano *et al.* 1997), and *COX-2*-null embryonic stem cells have a dramatically reduced ability to form teratomas when injected into syngeneic mice (Zhang *et al.* 2000*a*). Interestingly, disruption of either *COX-1* or *COX-2* caused similar reductions in tumour multiplicity in the Min mouse (Chulada *et al.* 2000), suggesting that both enzymes can impact on tumorigenesis. The results of similar studies to determine the effects of *COX-2* deficiency on the incidence of mammary cancer are eagerly awaited. However, results from the converse experiment designed to address the consequence of *COX-2* overexpression in mammary gland have recently been reported (Liu *et al.* 2001). Liu and colleagues overexpressed human *COX-2* from the mouse mammary tumor virus (MMTV) promoter, and demonstrated that *COX-2* overexpression was sufficient to cause breast tumour formation in more than 85% of multiparous mice. Virgin females did not develop tumours, but exhibited precocious lobuloalveolar differentiation and enhanced expression of the milk protein β-casein. MMTV-driven *COX-2* expression increased during pregnancy, suggesting a basis for the failure of virgin animals to develop tumours. Interestingly, mammary gland involution was delayed in *COX-2* transgenic mice, with a decrease in the apoptotic index of mammary epithelial cells, and *COX-2*-induced tumor tissue expressed

reduced levels of the proapoptotic proteins Bax and Bcl-x_L. Together, these observations suggest that *COX-2* expression may contribute to tumorigenesis via a reduction in apoptosis, a result previously suggested by *in vitro* studies, as discussed below.

Pharmacological studies in rodent models of intestinal tumorigenesis

In addition to genetic evidence implicating cyclooxygenases in intestinal tumorigenesis, there are complementary pharmacological data. Many animal-based studies have been performed to investigate the utility of cyclooxygenase inhibitors for prevention or treatment of intestinal tumours. The prevention studies have predominantly examined either AOM-induced lesions in rat colon (aberrant crypt foci or carcinomas) or intestinal adenomas in *Apc*-deficient mice. A consistent finding has been that tumour incidence and multiplicity are reduced by both nonselective NSAIDs (Reddy *et al.* 1993, Rao *et al.* 1995, Boolbol *et al.* 1996, Jacoby *et al.* 1996, 2000*a*), and selective COX-2 inhibitors (Table 3). In addition, those tumours that do develop in drug-treated animals tend to be reduced in size relative to those in control animals (Nakatsugi *et al.* 1997, Fukutake *et al.* 1998, Jacoby *et al.* 2000*a,b*, Reddy *et al.* 2000*a*). It is notable that selective COX-2 inhibitors appear to be at least as effective in preventing tumours as nonselective NSAIDs. This result has important clinical implications, given the enhanced safety profile of selective COX-2 inhibitors versus traditional NSAIDs.

In addition to these prevention studies, cyclooxygenase inhibitors are also being evaluated as therapeutic agents for pre-existing tumours. Reduction in growth of colon cancer xenografts has been achieved by treatment with meloxicam, SC-58125 and celecoxib (Sheng *et al.* 1997, Goldman *et al.* 1998, Williams *et al.* 2000*b*). Celecoxib also decreased tumour multiplicity in Min mice by 52%, when administered after adenomas had been established (Jacoby *et al.* 2000*b*).

Pharmacological studies in rat breast cancer models

Carcinogen-induced rat mammary tumours have been used as a model system to test various NSAIDs and, more recently, selective COX-2 inhibitors for their chemopreventive potential (Table 4). In general, indomethacin was found to reduce the incidence and multiplicity of DMBA-induced tumours (Carter *et al.* 1983, 1989, McCormick *et al.* 1985, Noguchi *et al.* 1991). Because the incidence of breast cancer may be affected by dietary fat, some of these studies have compared NSAID effects in cohorts of animals fed low- versus high-fat diets. Carter *et al.* (1983) found that indomethacin reduced tumour incidence in DMBA-treated animals fed 18% corn oil to the level observed in DMBA-treated animals fed 5% corn oil, but did not see an effect on incidence in the low-fat cohort. In contrast, two other studies found that the inhibitory effect of indomethacin was not confined to rats fed high-fat diets (McCormick *et al.* 1985, Noguchi *et al.* 1991). Interestingly, McCormick *et al.* (1985) found that indomethacin treatment from 2 weeks before to 1 week after DMBA administration primarily targeted benign tumours. However, when treatment with indomethacin was initiated 1 week after DMBA and continued until the end of the trial, the multiplicity of malignant tumours was also significantly reduced. Conflicting data were obtained by Abou-el-Ela *et al.* (1989) who found no inhibition of mammary tumorigenesis by indomethacin. The basis for these discrepant observations is unclear. Two additional NSAIDs, flurbiprofen and aspirin, are also capable of reducing carcinogen-induced mammary tumorigenesis (McCormick & Moon 1983, Suzui *et al.* 1997, Mori *et al.* 1999), although piroxicam was not found to be effective in one study (Kitagawa & Noguchi 1994).

Two recent studies evaluated the effects of selective COX-2 inhibitors on mammary tumorigenesis. As shown in Fig. 4A, treatment with celecoxib significantly delayed tumour onset in DMBA-treated rats, and was more effective than ibuprofen (Harris *et al.* 2000). Dietary administration

Table 3 Chemoprevention of intestinal tumorigenesis in rodents using selective COX-2 inhibitors.

Reference	Animal	Model	Tumour type	Drug	Effect on tumour multiplicity
Oshima *et al.* (1996)	Mouse	$Apc^{\Delta 716}$	Adenoma	MF tricyclic	62% inhibition
Nakatsugi *et al.* (1997)	Mouse	Apc Min	Adenoma	Nimesulide	48% inhibition
Kawamori *et al.* (1998)	Rat	AOM	Colon carcinoma	Celecoxib	97% inhibition
Fukutake *et al.* (1998)	Mouse	AOM	Colon carcinoma	Nimesulide	81% inhibition
Reddy *et al.* (2000*a*)	Rat	AOM	Colon carcinoma	Celecoxib	84% inhibition
Sasai *et al.* (2000)	Mouse	$Apc^{\Delta 474}$	Adenoma	JTE-522	32% inhibition
Jacoby *et al.* (2000*b*)	Mouse	Apc Min	Adenoma	Celecoxib	71% inhibition

Several of these studies tested a range of drug concentrations. The effect on tumour multiplicity (number of tumours per animal) reported in this table was that achieved at the highest drug dose tested. In addition to inhibition of tumour multiplicity, these agents also caused reduced tumour incidence (proportion of animals with tumours). Note that individual studies examined different endpoints – carcinomas or adenomas.

Table 4 Chemoprevention of mammary tumorigenesis in rats using cyclooxygenase inhibitors

Reference	Tumour induction	Drug	Effects
Carter *et al.* (1983)	DMBA/18% corn oil	Indomethacin	54% inhibition of tumour multiplicity; reduction in tumour incidence
McCormick & Moon (1983)	NMU	Flurbiprofen	Reduction in tumour incidence and multiplicity at low NMU dose
McCormick *et al.* (1985)	DMBA	Indomethacin	Reduction in benign or malignant tumours according to period of drug administration
Abou-el-Ela *et al.* (1989)	DMBA	Indomethacin	No inhibition
Carter *et al.* (1989)	DMBA/20% fat	Indomethacin	Inhibition of tumorigenesis in animals fed 4 or 12% linoleate
Noguchi *et al.* (1991)	DMBA/20% corn oil	Indomethacin	61% inhibition of tumour multiplicity; reduction in tumour incidence
Kitagawa & Noguchi (1994)	DMBA/20% soybean oil	Piroxicam	No inhibition
Suzui *et al.* (1997)	PhIP/high corn oil	Aspirin	44% inhibition of tumour multiplicity
Mori *et al.* (1999)	PhIP/high fat	Aspirin	Inhibited tumour multiplicity
Harris *et al.* (2000)	DMBA	Celecoxib	86% inhibition of tumour multiplicity; 68% reduction in tumour incidence
Nakatsugi *et al.* (2000)	PhIP/24% corn oil	Nimesulide	54% inhibition of tumour multiplicity; 28% reduction in tumour incidence

of celecoxib reduced incidence, multiplicity and volume of malignant breast tumours by 68%, 86% and 81% respectively relative to the control group. The chemopreventive properties of another COX-2 inhibitor, nimesulide, was tested in rats in which the environmental carcinogen PhIP, together with a 24% corn oil diet, was used to induce *COX-2* expression and mammary tumours (Nakatsugi *et al.* 2000). A small reduction in tumour incidence was achieved by administration of 400 parts per million nimesulide (Table 5). In addition, both size and multiplicity of tumours were significantly reduced in the nimesulide-treated animals. Together, these studies represent the first direct evidence that selective COX-2 inhibitors can protect against experimental breast cancer.

Two additional studies suggest that cyclooxygenase inhibition may be a useful strategy for treating breast cancer. Robertson *et al.* (1998) measured tumour size in rats that were maintained for 100 days post DMBA treatment then fed a control or an ibuprofen-containing diet for an additional 5 weeks prior to necropsy. Tumours from the control animals increased in volume by approximately 180%. In contrast, those from the ibuprofen-treated cohort decreased in volume by almost 40%. More recently, a similar study was conducted in which the effects of the selective COX-2 inhibitor celecoxib were investigated (Alshafie *et al.* 2000). In this study, rats were maintained for 4 months post DMBA treatment to induce tumours. Subsequently, the rats were given a control or a celecoxib-containing diet for an additional 6 weeks. The mean tumour volume increased by 518% in control animals, but decreased by 32% in the group fed celecoxib (Fig. 4B). In addition, the total tumour number continued to increase in the control animals, but was reduced in the celecoxib cohort. This report of regression of mammary tumours *in vivo* by a selective COX-2 inhibitor is consistent with earlier studies showing that various NSAIDs reduced the growth of mammary tumour xenografts (Fulton 1984, Karmali & Marsh 1986). Together, these observations suggest that COX-2 inhibition may represent a strategy not only for prevention but also for treatment of human breast cancer.

The ability of COX-2 inhibitors to significantly reduce tumour multiplicity strongly suggests that COX-2 contributes to tumorigenesis. However, COX-independent effects of NSAIDs have also been described (see below), raising the possibility that the observed inhibition may not necessarily be ascribed solely to effects on COX-2. Nevertheless, taken together the pharmacological and genetic studies provide overwhelming support for a role for COX-2 in tumorigenesis. Definitive evidence has now been provided by the recent demonstration that *COX-2* overexpression is sufficient to induce mammary tumor formation in transgenic mice (Liu *et al.* 2001).

How does COX-2 contribute to cancer?

Prostaglandins stimulate proliferation and mediate immune suppression

Since COX-2 is a PG synthase, the most obvious consequence of *COX-2* overexpression is increased PG production, and indeed high PG levels have been detected in many cancers. Enhanced PG synthesis may contribute to carcinogenesis in several ways, including direct stimulation of cell growth. $PGE_{2\alpha}$ and $PGF_{2\alpha}$ can both stimulate mitogenesis in Balb/c 3T3 fibroblasts in synergy with epidermal growth factor (EGF) (Nolan *et al.* 1988), and $PGF_{2\alpha}$ is also mitogenic for Swiss 3T3 cells and osteoblasts

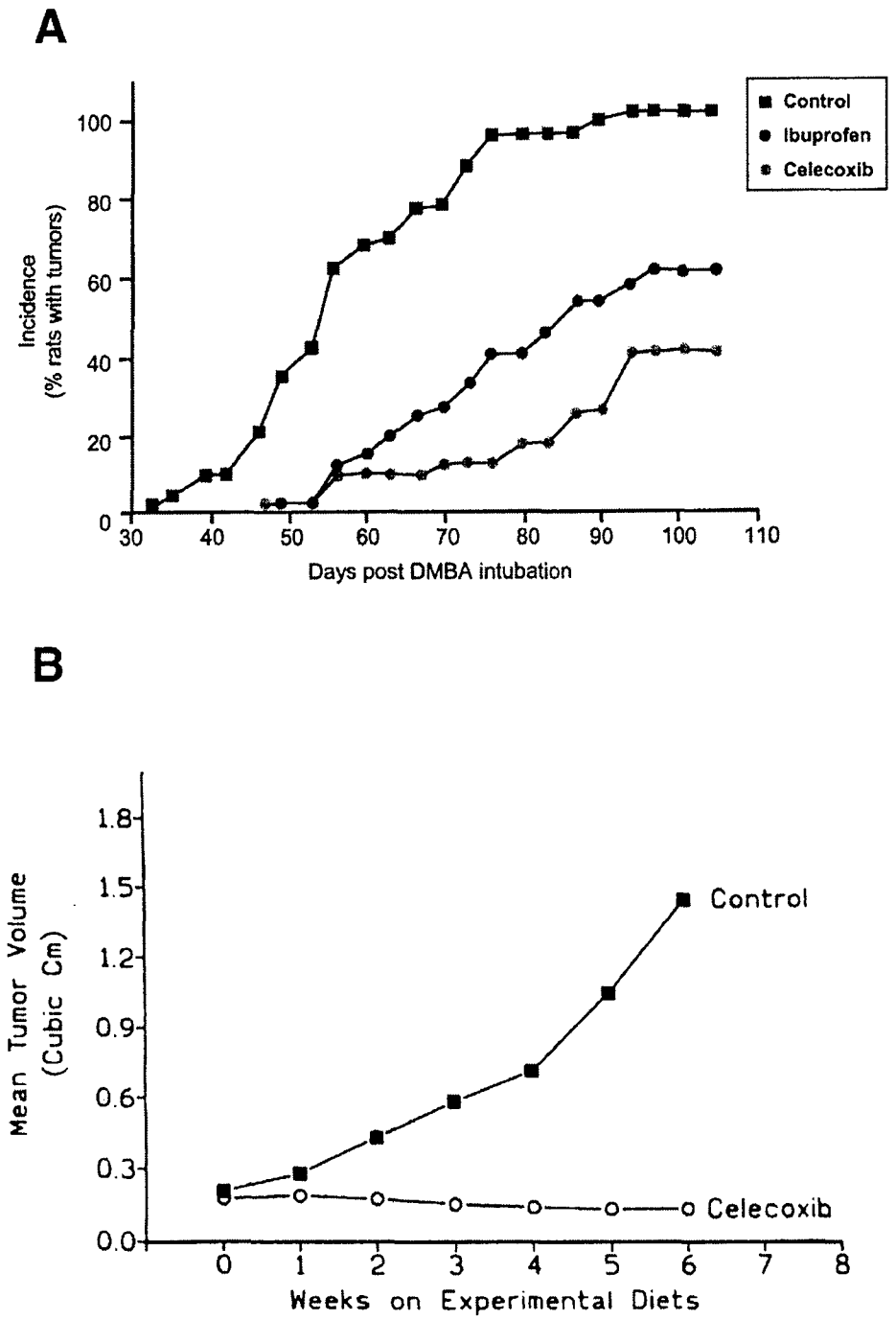

Figure 4 Celecoxib is effective for breast cancer prevention and treatment. (A) Rats were assigned to a control diet, or a diet containing 1500 ppm ibuprofen or 1500 ppm celecoxib 7 days prior to a single intragastric dose of DMBA, and tumour incidence was measured for 16 weeks. This figure is reproduced with permission from Harris *et al.* (2000). (B) Rats were maintained for four months after a single intragastric dose of DMBA to allow palpable tumour development, then assigned to a control diet or a diet containing 1500 ppm celecoxib, and tumour size was monitored for 6 weeks. This figure is reproduced with permission from Alshafie *et al.* (2000).

Table 5 Effects of nimesulide on the incidence, multiplicity and volume of mammary carcinomas induced by PhIP in Sprague-Dawley rats. Results are means ± S.E.

	Control diet	400 ppm nimesulide
Tumour incidence (% rats with cancers)	30/42 (71%)	19/37 (51%)
Multiplicity (no. of cancers/rat)	2.6 ± 0.5	1.2 ± 0.2*
Cancer volume/rat (cm^3)	4.1 ± 1.3	1.1 ± 0.4*
Effective no. of rats	42	37

*Significantly different from the control diet group by Welch's *t* test ($P > 0.05$).
This table is reproduced with permission from Nakatsugi *et al.* (2000).

(Goin *et al.* 1993, Quarles *et al.* 1993). Both PGE_1 and PGE_2 stimulate proliferation of mammary epithelial cells in the presence of EGF (Bandyopadhyay *et al.* 1987). Thus, inappropriate stimulation of cellular proliferation by PGs may contribute to tumorigenesis. However, PGs do not act as mitogens for all cell types, and in fact depress proliferation of some cells, particularly those of the immune system (Marnett 1992).

Antiproliferative effects may contribute to the immune suppression associated with PGs. PGE_2 inhibits T and B cell proliferation and cytokine synthesis, and diminishes the cytotoxic activity of natural killer cells. PGE_2 also inhibits the production of TNFα while inducing interleukin-10 production, which itself has immunosuppressive effects (Huang *et al.* 1996). PGs may also inhibit antigen processing by dendritic cells (Stolina *et al.* 2000). Thus, PG-mediated immune suppression may contribute to tumorigenesis, since this may allow tumours to avoid immune surveillance that might otherwise limit their growth.

In breast tissue, PGs may also stimulate proliferation indirectly by increasing oestrogen biosynthesis (Harris *et al.* 1999). The aromatase gene *CYP19*, which is responsible for oestrogen synthesis, has three promoters, I.4, I.3 and II, from which distinct transcripts are generated. In adipose tissue, aromatase is normally expressed from promoter I.4. However, in adipose tissue adjacent to breast tumours, *CYP19* tends to be expressed from promoter II. Recently, PGE_2 has been demonstrated to increase aromatase activity (Zhao *et al.* 1996, Purohit *et al.* 1999) and cause *CYP19* promoter switching to promoter II in adipose stromal cells (Zhao *et al.* 1996). These data suggest that PG overproduction can induce aromatase, leading to increased oestrogen synthesis. Consistent with this, a positive correlation has been observed between *CYP19* and *COX* expression in human breast cancer specimens (Brueggemeier *et al.* 1999). Thus it is possible that PG-mediated oestrogen overproduction may be an important organ site-specific consequence of *COX-2* upregulation in breast cancer.

Cyclooxygenase-mediated production of mutagens

Thus far, the potential contributions of PG overproduction to tumorigenesis, including increased cellular proliferation and diminished immune surveillance, have been discussed. However, *COX-2* overexpression may also have PG-independent consequences. In particular, *COX-2* overexpression may result in increased production of mutagens. Malondialdehyde (MDA) can be produced by isomerisation of PGH_2 both enzymatically and non-enzymatically (Fig. 1). Therefore, MDA production may be elevated due to increased availability of the precursor molecule PGH_2. MDA forms adducts with deoxynucleosides and induces frame-shifts and base-pair substitutions, and thus has potent mutagenic activity (Marnett 1992). Additional carcinogens can be formed by oxidation of aromatic amines, heterocyclic amines, and dihydrodiol derivatives of polycyclic hydrocarbons (Wiese *et al.* 2001). This oxidation step is catalysed by the peroxidase activity of cyclooxygenase, which requires a reductant to convert PGG_2 to PGH_2. Thus, *COX-2* overexpression may lead to DNA damage, thereby contributing to carcinogenesis. Consistent with this hypothesis, the selective COX-2 inhibitor nimesulide decreases formation of the mutagen 8-oxo-7,8-dihydro-2′-deoxyguanosine in the colonic mucosa (Tardieu *et al.* 2000).

Effects on angiogenesis

Recently, it has become apparent that cyclooxygenases are involved in angiogenesis (reviewed by Gately 2000). This is a crucial facet of tumorigenesis since neovascularisation is required for tumours to grow beyond 2–3 mm in size. Experiments in the 1980s showed that xenograft vascularisation was significantly reduced by the NSAIDs indomethacin, diclofenac and aspirin (Peterson 1983). More recently, COX-2 has been specifically implicated. *In vitro*, selective COX-2 inhibitors decrease endothelial tubule formation (Tsujii *et al.* 1998, Jones *et al.* 1999), while, *in vivo*, selective COX-2 inhibitors reduce angiogenesis in several models (Majima *et al.* 1997, Daniel *et al.* 1999, Sawaoka *et al.* 1999, Yamada *et al.* 1999, Masferrer *et al.* 2000). A representative illustration of celecoxib-mediated inhibition of corneal angiogenesis is shown in Fig. 5.

In an interesting corollary, Lewis lung carcinoma xenografts showed marked attenuation of growth when implanted in *COX-2*-null mice, but grew normally in *COX-1*-deficient mice (Williams *et al.* 2000*a*). The tumours from *COX-2* knockout mice exhibited 30% decreased vascular density, implicating host COX-2 in tumour neovascularisation. It seems likely that COX-2 in epithelial cells, endothelial cells and fibroblasts may all contribute to the angiogenic process, although there are some

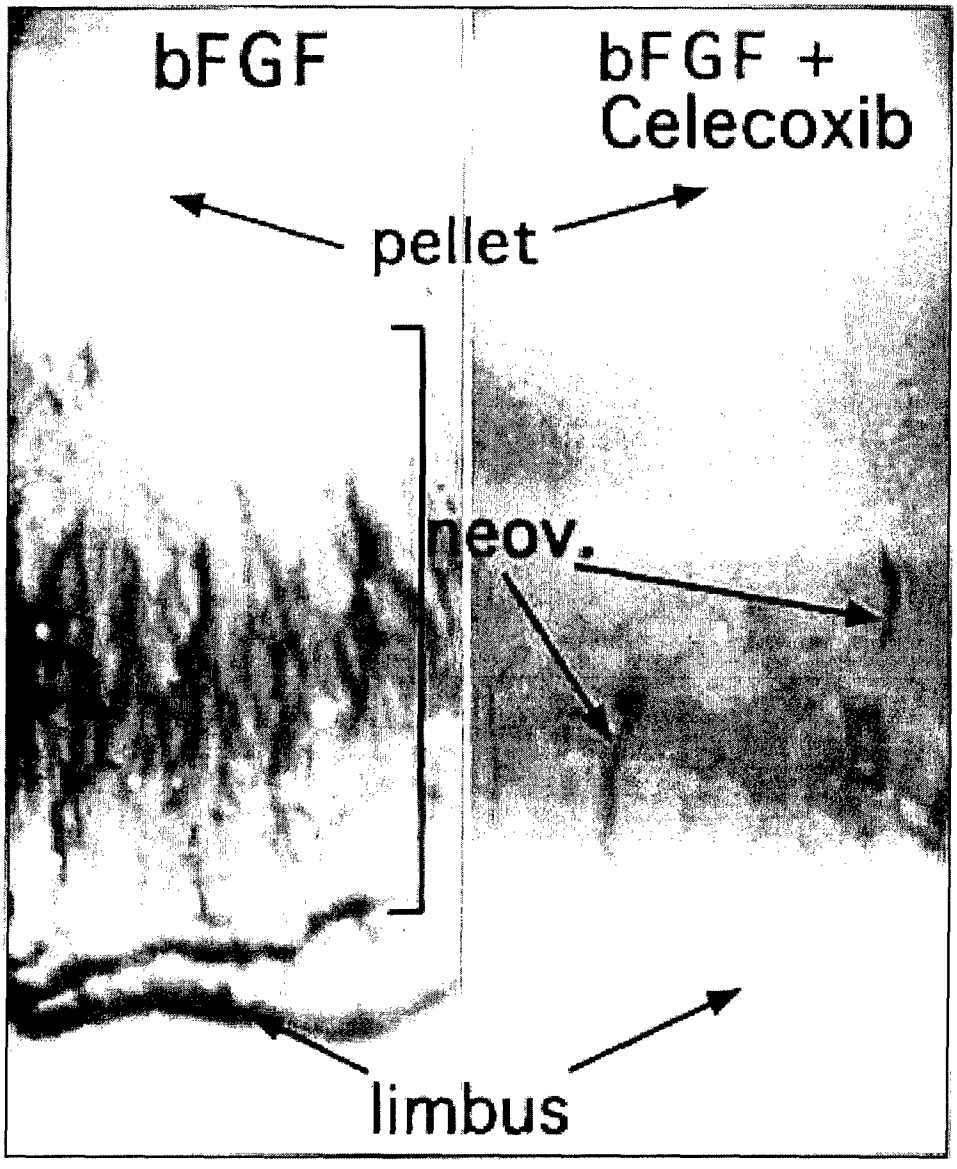

Figure 5 Celecoxib inhibits corneal angiogenesis induced by bFGF. The contribution of COX-2 to angiogenesis was evaluated in an *in vivo* rat corneal model. Implanted bFGF induced neovascularisation (neov.; left panel) accompanied by corneal thickening. Celecoxib caused a substantial reduction in the number and length of sprouting capillaries (right panel). This figure is reproduced with permission from Masferrer *et al.* (2000).

discrepancies between observations made *in vivo* and *in vitro* (Majima *et al.* 1997, Tsujii *et al.* 1998, Daniel *et al.* 1999, Masferrer *et al.* 2000, Williams *et al.* 2000*a*). COX-2 apparently contributes to the production of pro-angiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor-1, PDGF, and endothelin-1. NS-398 treatment of a *COX-2*-overexpressing colorectal cancer cell line diminishes secretion of these factors (Tsujii *et al.* 1998), and *COX-2* (–/–) fibroblasts have a 94% reduction in the ability to produce VEGF relative to wild-type fibroblasts (Williams *et al.* 2000*a*). However, the molecular mechanisms underlying COX-2-mediated production of pro-angiogenic factors remain to be defined.

COX-1 can also contribute to angiogenesis. Nonselective NSAIDs decrease the vascularisation of xenografts comprised of cells not expressing *COX-2* (Sawaoka *et al.* 1999). Moreover, NSAIDs inhibit endothelial tubule formation even when cells do not express *COX-2* (Tsujii *et al.* 1998, Jones *et al.* 1999). Thus both COX-1 and COX-2 are likely to contribute to tumour vascularisation. The possibility that COX-2 inhibitors diminish tumorigenesis

partly by preventing angiogenesis further enhances their attractiveness as potential anti-cancer agents.

Effects of *COX-2* overexpression on cell invasiveness and apoptosis

The potential consequences of *COX-2* overexpression have been addressed *in vitro* by generation of cell lines overexpressing *COX-2*. In particular, rat intestinal epithelial cells stably overexpressing *COX-2* show several altered characteristics, including increased adhesion to extracellular matrix, resistance to butyrate-induced apoptosis and a delayed transit through the G1 phase of the cell cycle (Tsujii & DuBois 1995, DuBois *et al.* 1996*b*). Additionally, stable *COX-2* expression in Caco-2 cells or in the breast cancer cell line Hs578T increases expression or activity of enzymes capable of digesting the basement membrane, presumably contributing to the observed increase in ability to invade through a layer of Matrigel (Tsujii *et al.* 1997, Takahashi *et al.* 1999). All of these characteristics may contribute to tumorigenicity, although the molecular mechanism(s) by which COX-2 causes these effects is unknown.

Much interest has centred on the ability of COX-2 to suppress apoptosis. Diminished apoptosis is thought to favour carcinogenesis by permitting survival of cells that have acquired mutations, and thus is viewed as one of the central mechanisms of tumorigenesis. Conversely, many NSAIDs enhance apoptotic cell death, although this is unlikely to be solely due to inhibition of cyclooxygenase activity (see below). Several hypotheses have been advanced to account for suppression of apoptosis in response to *COX-2* overexpression. The ability of PGE_2 to inhibit apoptosis caused by a selective COX-2 inhibitor, and concomitantly to induce *Bcl-2*, suggests that PG-mediated upregulation of *Bcl-2* may suppress apoptosis (Sheng *et al.* 1998*a*). Alternatively, since arachidonic acid stimulates apoptosis, enhanced *COX-2* expression could inhibit apoptosis by increasing the conversion of arachidonic acid to PG (Chan *et al.* 1998, Cao *et al.* 2000). Kinzler and colleagues propose that arachidonic acid stimulates the conversion of sphingomyelin to ceramide, which then causes apoptosis (Chan *et al.* 1998). They further suggest that the apoptosis-promoting effect of NSAIDs such as sulindac is due to NSAID-induced accumulation of arachidonic acid. In contrast, although Prescott and co-workers also consider arachidonate to be a key determinant of apoptosis, they do not observe increased levels of ceramide in response to exogenous administration of arachidonic acid (Cao *et al.* 2000).

Clearly, the suppression of apoptosis associated with *COX-2* overexpression could be an important factor in tumorigenesis, although the precise mechanistic basis remains uncertain. Interestingly, an apoptosis-related protein was found in a two-hybrid screen designed to identify proteins that interact with cyclooxygenases (Ballif *et al.* 1996). Nucleobindin associates with DNA from apoptotic cells, and can itself promote apoptosis. The interaction of COX-1 and COX-2 with nucleobindin may contribute to COX-mediated suppression of apoptosis, potentially via sequestration of nucleobindin, but further studies are required to fully understand the significance of the interaction.

As mentioned above, multiple NSAIDs, including selective COX-2 inhibitors, induce apoptosis in a variety of cells (Lu *et al.* 1995, Hara *et al.* 1997, Sheng *et al.* 1998a, Ding *et al.* 2000, Hida *et al.* 2000, Li *et al.* 2000). The simplest interpretation of this phenomenon is that, since *COX-2* overexpression suppresses apoptosis, inhibition of COX-2 activity is sufficient to induce apoptosis. However, NSAID-induced apoptosis has also been demonstrated in cell lines that do not express *COX-2*, including *COX-2*-null mouse embryo fibroblasts (Hanif *et al.* 1996, Elder *et al.* 1997, Zhang *et al.* 1999). Additionally, non-cyclooxygenase-inhibiting sulindac metabolites such as sulindac sulphone retain the ability to induce apoptosis (Piazza *et al.* 1997, Lim *et al.* 1999*b*). Thus, NSAIDs most likely stimulate apoptosis via both COX-dependent and -independent mechanisms (Rigas & Shiff 2000), including inhibition of the protein kinase Akt (Hsu *et al.* 2000) and suppression of NF-κB activation (Kopp & Ghosh 1994, Grilli *et al.* 1996, Yin *et al.* 1998, Yamamoto *et al.* 1999).

Clinical prospects for COX-2 inhibitors and breast cancer

The weight of evidence implicating COX-2 in colorectal cancer has stimulated clinical trials to investigate the efficacy of selective COX-2 inhibitors in individuals at risk for colorectal cancer. Treatment with celecoxib has been shown to reduce the size and number of polyps in FAP patients (Steinbach *et al.* 2000), and is currently being evaluated for efficacy in preventing sporadic colorectal adenomas. Undoubtedly the potential use of selective COX-2 inhibitors for the treatment of colorectal cancer will also be investigated.

Here we have reviewed evidence that aberrant *COX-2* expression is also associated with breast cancer, both in rodent models and in the human disease. Selective COX-2 inhibitors have proved effective in preventing experimental breast cancer (Harris *et al.* 2000, Nakatsugi *et al.* 2000). Whether COX-2 inhibitors will also be useful for preventing breast cancer in high-risk individuals needs to be investigated. In addition, selective COX-2 inhibitors may have a role in the treatment of breast cancer (Alshafie *et al.* 2000). Since *COX-2* is overexpressed in HER-2/neu-positive breast cancers (Subbaramaiah *et al.* 1999*b*), selective COX-2 inhibitors should be evaluated as therapy in this patient population. Because COX-2-derived PGs may enhance aromatase activity, a therapeutic regimen combining a selective COX-2 inhibitor with an aromatase inhibitor should be considered. There is also recent evidence that

microtubule-interfering agents, including taxol, stimulate *COX-2* transcription (Subbaramaiah *et al.* 2000). This could decrease the efficacy of this class of drugs. Thus, coadministration of a selective COX-2 inhibitor with drugs such as taxol might enhance their anti-cancer activity. Finally, a number of natural substances have been identified that inhibit the transcriptional activation of *COX-2*. Examples include retinoids, triterpenoids, antioxidants and resorcins (Mestre *et al.* 1997*a,b*, Chinery *et al.* 1998, Subbaramaiah *et al.* 1998*b*, Suh *et al.* 1998, Mutoh *et al.* 2000). Some of these compounds also inhibit experimental breast cancer. Hence, it is possible that studies of COX-2 will provide insights that will prove useful in developing dietary recommendations to decrease cancer risk.

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References

Abou-el-Ela SH, Prasse KW, Farrell RL, Carroll RW, Wade AE & Bunce OR 1989 Effects of D,L-2-difluoromethylornithine and indomethacin on mammary tumor promotion in rats fed high n-3 and/or n-6 fat diets. *Cancer Research* **49** 1434–1440.

Alshafie GA, Abou-Issa HM, Seibert K & Harris RE 2000 Chemotherapeutic evaluation of celecoxib, a cyclooxygenase-2 inhibitor, in a rat mammary tumor model. *Oncology Reports* **7** 1377–1381.

Badawi AF & Archer MC 1998 Effect of hormonal status on the expression of the cyclooxygenase 1 and 2 genes and prostaglandin synthesis in rat mammary glands. *Prostaglandins and Other Lipid Mediators* **56** 167–181.

Badawi AF, El-Sohemy A, Stephen LL, Ghoshal AK & Archer MC 1998 The effect of dietary n-3 and n-6 polyunsaturated fatty acids on the expression of cyclooxygenase 1 and 2 and levels of p21ras in rat mammary glands. *Carcinogenesis* **19** 905–910.

Badawi AF, el-Sohemy A, Stephen LL, Ghoshal AK & Archer MC 1999 Modulation of the expression of the cyclooxygenase 1 and 2 genes in rat mammary glands: role of hormonal status and dietary fat. *Advances in Experimental Medicine and Biology* **469** 119–124.

Ballif BA, Mincek NV, Barratt JT, Wilson ML & Simmons DL 1996 Interaction of cyclooxygenases with an apoptosis- and autoimmunity-associated protein. *PNAS* **93** 5544–5549.

Bandyopadhyay GK, Imagawa W, Wallace D & Nandi S 1987 Linoleate metabolites enhance the *in vitro* proliferative response of mouse mammary epithelial cells to epidermal growth factor. *Journal of Biological Chemistry* **262** 2750–2756.

Bennett A, Charlier EM, McDonald AM, Simpson JS, Stamford IF & Zebro T 1977 Prostaglandins and breast cancer. *Lancet* **2** 624–626.

Boolbol SK, Dannenberg AJ, Chadburn A, Martucci C, Guo XJ, Ramonetti JT, Abreu-Goris M, Newmark HL, Lipkin ML, DeCosse JJ & Bertagnolli MM 1996 Cyclooxygenase-2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis. *Cancer Research* **56** 2556–2560.

Brueggemeier RW, Quinn AL, Parrett ML, Joarder FS, Harris RE & Robertson FM 1999 Correlation of aromatase and cyclooxygenase gene expression in human breast cancer specimens. *Cancer Letters* **140** 27–35.

Buckman SY, Gresham A, Hale P, Hruza G, Anast J, Masferrer J & Pentland AP 1998 COX-2 expression is induced by UVB exposure in human skin: implications for the development of skin cancer. *Carcinogenesis* **19** 723–729.

Cao Y, Pearman AT, Zimmerman GA, McIntyre TM & Prescott SM 2000 Intracellular unesterified arachidonic acid signals apoptosis. *PNAS* **97** 11280–11285.

Carter CA, Milholland RJ, Shea W & Ip MM 1983 Effect of the prostaglandin synthetase inhibitor indomethacin on 7,12-dimethylbenz(a)anthracene-induced mammary tumorigenesis in rats fed different levels of fat. *Cancer Research* **43** 3559–3562.

Carter CA, Ip MM & Ip C 1989 A comparison of the effects of the prostaglandin synthesis inhibitors indomethacin and carprofen on 7,12-dimethylbenz[a]anthracene-induced mammary tumorigenesis in rats fed different amounts of essential fatty acid. *Carcinogenesis* **10** 1369–1374.

Chan G, Boyle JO, Yang EK, Zhang F, Sacks PG, Shah JP, Edelstein D, Soslow RA, Koki AT, Woerner BM, Masferrer JL & Dannenberg AJ 1999 Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. *Cancer Research* **59** 991–994.

Chan TA, Morin PJ, Vogelstein B & Kinzler KW 1998 Mechanisms underlying nonsteroidal antiinflammatory drug-mediated apoptosis. *PNAS* **95** 681–686.

Chinery R, Beauchamp RD, Shyr Y, Kirkland SC, Coffey RJ & Morrow JD 1998 Antioxidants reduce cyclooxygenase-2 expression, prostaglandin production, and proliferation in colorectal cancer cells. *Cancer Research* **58** 2323–2327.

Chulada PC, Thompson MB, Mahler JF, Doyle CM, Gaul BW, Lee C, Tiano HF, Morham SG, Smithies O & Langenbach R 2000 Genetic disruption of Ptgs-1, as well as Ptgs-2, reduces intestinal tumorigenesis in Min mice. *Cancer Research* **60** 4705–4708.

Crawford HC, Fingleton B, Gustavson MD, Wagenaar RA, Hassell JA & Matrisian LM 2001 The PEA3 subfamily of Ets transcription factors synergizes with β-catenin/LEF-1 to activate matrilysin transcription in intestinal tumors. *Molecular and Cellular Biology* **21** 1370–1383.

Daniel TO, Liu H, Morrow JD, Crews BC & Marnett LJ 1999 Thromboxane A2 is a mediator of cyclooxygenase-2-dependent endothelial migration and angiogenesis. *Cancer Research* **59** 4574–4577.

Dinchuk JE, Car BD, Focht RJ, Johnston JJ, Jaffee BD, Covington MB, Contel NR, Eng VM, Collins RJ, Czerniak PM, Stewart AG & Trzaskos JM 1995 Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. *Nature* **378** 406–409.

Ding XZ, Tong WG & Adrian TE 2000 Blockade of cyclooxygenase-2 inhibits proliferation and induces apoptosis in

human pancreatic cancer cells. *Anticancer Research* **20** 2625–2631.

Dixon DA, Kaplan CD, McIntyre TM, Zimmerman GA & Prescott SM 2000 Post-transcriptional control of cyclooxygenase-2 gene expression. The role of the 3′-untranslated region. *Journal of Biological Chemistry* **275** 11750–11757.

DuBois RN, Radhika A, Reddy BS & Entingh AJ 1996*a* Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors. *Gastroenterology* **110** 1259–1262.

DuBois RN, Shao J, Tsujii M, Sheng H & Beauchamp RD 1996*b* G_1 delay in cells overexpressing prostaglandin endoperoxide synthase-2. *Cancer Research* **56** 733–737.

Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S & DuBois RN 1994 Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* **107** 1183–1188.

Egan KM, Stampfer MJ, Giovannucci E, Rosner BA & Colditz GA 1996 Prospective study of regular aspirin use and the risk of breast cancer. *Journal of the National Cancer Institute* **88** 988–993.

Elder DJ, Halton DE, Hague A & Paraskeva C 1997 Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti-inflammatory drug: independence from COX-2 protein expression. *Clinical Cancer Research* **3** 1679–1683.

Fodde R, Edelmann W, Yang K, van Leeuwen C, Carlson C, Renault B, Breukel C, Alt E, Lipkin M, Khan PM *et al.* 1994 A targeted chain-termination mutation in the mouse *Apc* gene results in multiple intestinal tumors. *PNAS* **91** 8969–8973.

Friedman GD & Ury HK 1980 Initial screening for carcinogenicity of commonly used drugs. *Journal of the National Cancer Institute* **65** 723–733.

Fukutake M, Nakatsugi S, Isoi T, Takahashi M, Ohta T, Mamiya S, Taniguchi Y, Sato H, Fukuda K, Sugimura T & Wakabayashi K 1998 Suppressive effects of nimesulide, a selective inhibitor of cyclooxygenase-2, on azoxymethane-induced colon carcinogenesis in mice. *Carcinogenesis* **19** 1939–1942.

Fulton AM 1984 *In vivo* effects of indomethacin on the growth of murine mammary tumors. *Cancer Research* **44** 2416–2420.

Gately S 2000 The contributions of cyclooxygenase-2 to tumor angiogenesis. *Cancer and Metastasis Reviews* **19** 19–27.

Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, Booker SV, Robinson CR & Offerhaus GJ 1993 Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *New England Journal of Medicine* **328** 1313–1316.

Gilhooly EM & Rose DP 1999 The association between a mutated ras gene and cyclooxygenase-2 expression in human breast cancer cell lines. *International Journal of Oncology* **15** 267–270.

Goin M, Pignataro O & Jimenez de Asua L 1993 Early cell cycle diacylglycerol (DAG) content and protein kinase C (PKC) activity enhancement potentiates prostaglandin F2 alpha (PGF2 alpha) induced mitogenesis in Swiss 3T3 cells. *FEBS Letters* **316** 68–72.

Goldman AP, Williams CS, Sheng H, Lamps LW, Williams VP, Pairet M, Morrow JD & DuBois RN 1998 Meloxicam inhibits the growth of colorectal cancer cells. *Carcinogenesis* **19** 2195–2199.

Greenberg ER, Baron JA, Freeman DH Jr, Mandel JS & Haile R 1993 Reduced risk of large-bowel adenomas among aspirin users. The Polyp Prevention Study Group. *Journal of the National Cancer Institute* **85** 912–916.

Grilli M, Pizzi M, Memo M & Spano P 1996 Neuroprotection by aspirin and sodium salicylate through blockade of NF-kappaB activation. *Science* **274** 1383–1385.

Guerin M, Sheng ZM, Andrieu N & Riou G 1990 Strong association between c-myb and oestrogen-receptor expression in human breast cancer. *Oncogene* **5** 131–135.

Gupta S, Srivastava M, Ahmad N, Bostwick DG & Mukhtar H 2000 Over-expression of cyclooxygenase-2 in human prostate adenocarcinoma. *Prostate* **42** 73–78.

Haertel-Wiesmann M, Liang Y, Fantl WJ & Williams LT 2000 Regulation of cyclooxygenase-2 and periostin by Wnt-3 in mouse mammary epithelial cells. *Journal of Biological Chemistry* **275** 32046–32051.

Hamid R, Singh J, Reddy BS & Cohen LA 1999 Inhibition by dietary menhaden oil of cyclooxygenase-1 and -2 in N-nitrosomethylurea-induced rat mammary tumors. *International Journal of Oncology* **14** 523–528.

Hanif R, Pittas A, Feng Y, Koutsos MI, Qiao L, Staiano-Coico L, Shiff SI & Rigas B 1996 Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochemical Pharmacology* **52** 237–245.

Hara A, Yoshimi N, Niwa M, Ino N & Mori H 1997 Apoptosis induced by NS-398, a selective cyclooxygenase-2 inhibitor, in human colorectal cancer cell lines. *Japanese Journal of Cancer Research* **88** 600–604.

Harris RE, Namboodiri KK & Farrar WB 1996 Nonsteroidal antiinflammatory drugs and breast cancer. *Epidemiology* **7** 203–205.

Harris RE, Robertson FM, Abou-Issa HM, Farrar WB & Brueggemeier R 1999 Genetic induction and upregulation of cyclooxygenase (COX) and aromatase (CYP19): an extension of the dietary fat hypothesis of breast cancer. *Medical Hypotheses* **52** 291–292.

Harris RE, Alshafie GA, Abou-Issa H & Seibert K 2000 Chemoprevention of breast cancer in rats by celecoxib, a cyclooxygenase 2 inhibitor. *Cancer Research* **60** 2101–2103.

Herschman HR 1996 Prostaglandin synthase 2. *Biochimica et Biophysica Acta* **1299** 125–140.

Hida T, Yatabe Y, Achiwa H, Muramatsu H, Kozaki K, Nakamura S, Ogawa M, Mitsudomi T, Sugiura T & Takahashi T 1998 Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. *Cancer Research* **58** 3761–3764.

Hida T, Kozaki K, Muramatsu H, Masuda A, Shimizu S, Mitsudomi T, Sugiura T, Ogawa M & Takahashi T 2000 Cyclooxygenase-2 inhibitor induces apoptosis and enhances cytotoxicity of various anticancer agents in non-small cell lung cancer cell lines. *Clinical Cancer Research* **6** 2006–2011.

Howe LR, Subbaramaiah K, Chung WJ, Dannenberg AJ & Brown AMC 1999 Transcriptional activation of cyclooxygenase-2 in Wnt-1–transformed mouse mammary epithelial cells. *Cancer Research* **59** 1572–1577.

Howe LR, Crawford HC, Subbaramaiah K, Hassell JA, Dannenberg AJ & Brown AMC 2001 PEA3 is upregulated in response to Wnt1 and activates the expression of cyclooxygenase-2. *Journal of Biological Chemistry* (In Press).

Hsi LC, Angerman-Stewart J & Eling TE 1999 Introduction of full-length APC modulates cyclooxygenase-2 expression in HT-29 human colorectal carcinoma cells at the translational level. *Carcinogenesis* **20** 2045–2049.

Hsu AL, Ching TT, Wang DS, Song X, Rangnekar VM & Chen CS 2000 The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by blocking Akt activation in human prostate cancer cells independently of Bcl-2. *Journal of Biological Chemistry* **275** 11397–11403.

Huang M, Sharma S, Mao JT & Dubinett SM 1996 Non-small cell lung cancer-derived soluble mediators and prostaglandin E2 enhance peripheral blood lymphocyte IL-10 transcription and protein production. *Journal of Immunology* **157** 5512–5520.

Hwang D, Jang BC, Yu G & Boudreau M 1997 Expression of mitogen-inducible cyclooxygenase induced by lipopolysaccharide: mediation through both mitogen-activated protein kinase and NF-kappaB signaling pathways in macrophages. *Biochemical Pharmacology* **54** 87–96.

Hwang D, Scollard D, Byrne J & Levine E 1998 Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer. *Journal of the National Cancer Institute* **90** 455–460.

Inoue H, Yokoyama C, Hara S, Tone Y & Tanabe T 1995 Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. *Journal of Biological Chemistry* **270** 24965–24971.

Jacoby RF, Marshall DJ, Newton MA, Novakovic K, Tutsch K, Cole CE, Lubet RA, Kelloff GJ, Verma A, Moser AR & Dove WF 1996 Chemoprevention of spontaneous intestinal adenomas in the Apc Min mouse model by the nonsteroidal anti-inflammatory drug piroxicam. *Cancer Research* **56** 710–714.

Jacoby RF, Cole CE, Tutsch K, Newton MA, Kelloff G, Hawk ET & Lubet RA 2000*a* Chemopreventive efficacy of combined piroxicam and difluoromethylornithine treatment of Apc mutant Min mouse adenomas, and selective toxicity against Apc mutant embryos. *Cancer Research* **60** 1864–1870.

Jacoby RF, Seibert K, Cole CE, Kelloff G & Lubet RA 2000*b* The cyclooxygenase-2 inhibitor celecoxib is a potent preventive and therapeutic agent in the Min mouse model of adenomatous polyposis. *Cancer Research* **60** 5040–5044.

Jones MK, Wang H, Peskar BM, Levin E, Itani RM, Sarfeh IJ & Tarnawski AS 1999 Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing. *Nature Medicine* **5** 1418–1423.

Kargman SL, O'Neill GP, Vickers PJ, Evans JF, Mancini JA & Jothy S 1995 Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. *Cancer Research* **55** 2556–2559.

Karmali RA & Marsh J 1986 Antitumor activity in a rat mammary adenocarcinoma: the effect of cyclooxygenase inhibitors and immunization against prostaglandin E2. *Prostaglandins, Leukotrienes and Medicine* **23** 11–14.

Kawamori T, Rao CV, Seibert K & Reddy BS 1998 Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Research* **58** 409–412.

Kim Y & Fischer SM 1998 Transcriptional regulation of cyclooxygenase-2 in mouse skin carcinoma cells. Regulatory role of CCAAT/enhancer-binding proteins in the differential expression of cyclooxygenase-2 in normal and neoplastic tissues. *Journal of Biological Chemistry* **273** 27686–27694.

Kitagawa H & Noguchi M 1994 Comparative effects of piroxicam and esculetin on incidence, proliferation, and cell kinetics of mammary carcinomas induced by 7,12-dimethylbenz[a]anthracene in rats on high- and low-fat diets. *Oncology* **51** 401–410.

Koga H, Sakisaka S, Ohishi M, Kawaguchi T, Taniguchi E, Sasatomi K, Harada M, Kusaba T, Tanaka M, Kimura R, Nakashima Y, Nakashima O, Kojiro M, Kurohiji T & Sata M 1999 Expression of cyclooxygenase-2 in human hepatocellular carcinoma: relevance to tumor dedifferentiation. *Hepatology* **29** 688–696.

Kopp E & Ghosh S 1994 Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* **265** 956–959.

Kulkarni S, Rader JS, Zhang F, Liapis H, Koki AT, Masferrer JL, Subbaramaiah K & Dannenberg AJ 2001 Cyclooxygenase-2 is overexpressed in human cervical cancer. *Clinical Cancer Research* **7** 429–434.

Kutchera W, Jones DA, Matsunami N, Groden J, McIntyre TM, Zimmerman GA, White RL & Prescott SM 1996 Prostaglandin H synthase 2 is expressed abnormally in human colon cancer: evidence for a transcriptional effect. *PNAS* **93** 4816–4820.

Langman MJ, Jensen DM, Watson DJ, Harper SE, Zhao PL, Quan H, Bolognese JA & Simon TJ 1999 Adverse upper gastrointestinal effects of rofecoxib compared with NSAIDs. *Journal of the American Medical Association* **282** 1929–1933.

Li M, Lotan R, Levin B, Tahara E, Lippman SM & Xu XC 2000 Aspirin induction of apoptosis in esophageal cancer: a potential for chemoprevention. *Cancer Epidemiology, Biomarkers and Prevention* **9** 545–549.

Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM & Dey SK 1997 Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* **91** 197–208.

Lim H, Gupta RA, Ma WG, Paria BC, Moller DE, Morrow JD, DuBois RN, Trzaskos JM & Dey SK 1999*a* Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPARδ. *Genes and Development* **13** 1561–1574.

Lim JT, Piazza GA, Han EK, Delohery TM, Li H, Finn TS, Buttyan R, Yamamoto H, Sperl GJ, Brendel K, Gross PH, Pamukcu R & Weinstein IB 1999*b* Sulindac derivatives inhibit growth and induce apoptosis in human prostate cancer cell lines. *Biochemical Pharmacology* **58** 1097–1107.

Liu CH, Chang SH, Narko K, Trifan OC, Wu MT, Smith E, Haudenschild C, Lane TF & Hla T 2001 Overexpression of cyclooxygenase (Cox)-2 is sufficient to induce tumorigenesis in transgenic mice. *Journal of Biological Chemistry* (In Press).

Liu XH & Rose DP 1996 Differential expression and regulation of cyclooxygenase-1 and -2 in two human breast cancer cell lines. *Cancer Research* **56** 5125–5127.

Logan RF, Little J, Hawtin PG & Hardcastle JD 1993 Effect of aspirin and non-steroidal anti-inflammatory drugs on colorectal adenomas: case-control study of subjects participating in the Nottingham faecal occult blood screening programme. *British Medical Journal* **307** 285–289.

Lu X, Xie W, Reed D, Bradshaw WS & Simmons DL 1995 Nonsteroidal antiinflammatory drugs cause apoptosis and induce cyclooxygenases in chicken embryo fibroblasts. *PNAS* **92** 7961–7965.

McCormick DL & Moon RC 1983 Inhibition of mammary carcinogenesis by flurbiprofen, a non-steroidal antiinflammatory agent. *British Journal of Cancer* **48** 859–861.

McCormick DL, Madigan MJ & Moon RC 1985 Modulation of rat mammary carcinogenesis by indomethacin. *Cancer Research* **45** 1803–1808.

Majima M, Isono M, Ikeda Y, Hayashi I, Hatanaka K, Harada Y, Katsumata O, Yamashina S, Katori M & Yamamoto S 1997

Significant roles of inducible cyclooxygenase (COX)-2 in angiogenesis in rat sponge implants. *Japanese Journal of Pharmacology* **75** 105–114.

Marnett LJ 1992 Aspirin and the potential role of prostaglandins in colon cancer. *Cancer Research* **52** 5575–5589.

Masferrer JL, Leahy KM, Koki AT, Zweifel BS, Settle SL, Woerner BM, Edwards DA, Flickinger AG, Moore RJ & Seibert K 2000 Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. *Cancer Research* **60** 1306–1311.

Mestre JR, Subbaramaiah K, Sacks PG, Schantz SP, Tanabe T, Inoue H & Dannenberg AJ 1997*a* Retinoids suppress epidermal growth factor-induced transcription of cyclooxygenase-2 in human oral squamous carcinoma cells. *Cancer Research* **57** 2890–2895.

Mestre JR, Subbaramaiah K, Sacks PG, Schantz SP, Tanabe T, Inoue H & Dannenberg AJ 1997*b* Retinoids suppress phorbol ester-mediated induction of cyclooxygenase-2. *Cancer Research* **57** 1081–1085.

Mohammed SI, Knapp DW, Bostwick DG, Foster RS, Khan KN, Masferrer JL, Woerner BM, Snyder PW & Koki AT 1999 Expression of cyclooxygenase-2 (COX-2) in human invasive transitional cell carcinoma (TCC) of the urinary bladder. *Cancer Research* **59** 5647–5650.

Molina MA, Sitja-Arnau M, Lemoine MG, Frazier ML & Sinicrope FA 1999 Increased cyclooxygenase-2 expression in human pancreatic carcinomas and cell lines: growth inhibition by nonsteroidal anti-inflammatory drugs. *Cancer Research* **59** 4356–4362.

Morham SG, Langenbach R, Loftin CD, Tiano HF, Vouloumanos N, Jennette JC, Mahler JF, Kluckman KD, Ledford A, Lee CA & Smithies O 1995 Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell* **83** 473–482.

Mori H, Sugie S, Rahman W & Suzui N 1999 Chemoprevention of 2-amino-1–methyl-6-phenylimidazo [4,5-b]pyridine-induced mammary carcinogenesis in rats. *Cancer Letters* **143** 195–198.

Moser AR, Pitot HC & Dove WF 1990 A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* **247** 322–324.

Mutoh M, Takahashi M, Fukuda K, Matsushima-Hibiya Y, Mutoh H, Sugimura T & Wakabayashi K 2000 Suppression of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells by chemopreventive agents with a resorcin-type structure. *Carcinogenesis* **21** 959–963.

Nakatsugi S, Fukutake M, Takahashi M, Fukuda K, Isoi T, Taniguchi Y, Sugimura T & Wakabayashi K 1997 Suppression of intestinal polyp development by nimesulide, a selective cyclooxygenase-2 inhibitor, in Min mice. *Japanese Journal of Cancer Research* **88** 1117–1120.

Nakatsugi S, Ohta T, Kawamori T, Mutoh M, Tanigawa T, Watanabe K, Sugie S, Sugimura T & Wakabayashi K 2000 Chemoprevention by nimesulide, a selective cyclooxygenase-2 inhibitor, of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced mammary gland carcinogenesis in rats. *Japanese Journal of Cancer Research* **91** 886–892.

Noguchi M, Taniya T, Koyasaki N, Kumaki T, Miyazaki I & Mizukami Y 1991 Effects of the prostaglandin synthetase inhibitor indomethacin on tumorigenesis, tumor proliferation, cell kinetics, and receptor contents of 7,12-dimethylbenz(a)-anthracene-induced mammary carcinoma in Sprague-Dawley rats fed a high- or low-fat diet. *Cancer Research* **51** 2683–2689.

Nolan RD, Danilowicz RM & Eling TE 1988 Role of arachidonic acid metabolism in the mitogenic response of BALB/c 3T3 fibroblasts to epidermal growth factor. *Molecular Pharmacology* **33** 650–656.

Ochiai M, Oguri T, Isobe T, Ishioka S & Yamakido M 1999 Cyclooxygenase-2 (COX-2) mRNA expression levels in normal lung tissues and non-small cell lung cancers. *Japanese Journal of Cancer Research* **90** 1338–1343.

Okami J, Yamamoto H, Fujiwara Y, Tsujie M, Kondo M, Noura S, Oshima S, Nagano H, Dono K, Umeshita K, Ishikawa O, Sakon M, Matsuura N, Nakamori S & Monden M 1999 Overexpression of cyclooxygenase-2 in carcinoma of the pancreas. *Clinical Cancer Research* **5** 2018–2024.

Oshima M, Oshima H, Kitagawa K, Kobayashi M, Itakura C & Taketo M 1995 Loss of Apc heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated Apc gene. *PNAS* **92** 4482–4486.

Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans JF & Taketo MM 1996 Suppression of intestinal polyposis in $Apc^{\Delta 716}$ knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell* **87** 803–809.

Paganini-Hill A, Chao A, Ross RK & Henderson BE 1989 Aspirin use and chronic diseases: a cohort study of the elderly. *British Medical Journal* **299** 1247–1250.

Parrett ML, Harris RE, Joarder FS, Ross MS, Clausen KP & Robertson FM 1997 Cyclooxygenase-2 expression in human breast cancer. *International Journal of Oncology* **10** 503–507.

Peterson HI 1983 Effects of prostaglandin synthesis inhibitors on tumor growth and vascularization. Experimental studies in the rat. *Invasion and Metastasis* **3** 151–159.

Piazza GA, Rahm AK, Finn TS, Fryer BH, Li H, Stoumen AL, Pamukcu R & Ahnen DJ 1997 Apoptosis primarily accounts for the growth-inhibitory properties of sulindac metabolites and involves a mechanism that is independent of cyclooxygenase inhibition, cell cycle arrest, and p53 induction. *Cancer Research* **57** 2452–2459.

Purohit A, Singh A, Ghilchik MW & Reed MJ 1999 Inhibition of tumor necrosis factor α-stimulated aromatase activity by microtubule-stabilizing agents, paclitaxel and 2-methoxy-estradiol. *Biochemical and Biophysical Research Communications* **261** 214–217.

Quarles LD, Haupt DM, Davidai G & Middleton JP 1993 Prostaglandin F2 alpha-induced mitogenesis in MC3T3–E1 osteoblasts: role of protein kinase-C-mediated tyrosine phosphorylation. *Endocrinology* **132** 1505–1513.

Ramsay RG, Thompson MA, Hayman JA, Reid G, Gonda TJ & Whitehead RH 1992 Myb expression is higher in malignant human colonic carcinoma and premalignant adenomatous polyps than in normal mucosa. *Cell Growth and Differentiation* **3** 723–730.

Ramsay RG, Friend A, Vizantios Y, Freeman R, Sicurella C, Hammett F, Armes J & Venter D 2000 Cyclooxygenase-2, a colorectal cancer nonsteroidal anti-inflammatory drug target, is regulated by c-MYB. *Cancer Research* **60** 1805–1809.

Rao CV, Rivenson A, Simi B, Zang E, Kelloff G, Steele V & Reddy BS 1995 Chemoprevention of colon carcinogenesis by sulindac, a nonsteroidal anti-inflammatory agent. *Cancer Research* **55** 1464–1472.

Reddy BS, Rao CV, Rivenson A & Kelloff G 1993 Inhibitory effect of aspirin on azoxymethane-induced colon carcinogenesis in F344 rats. *Carcinogenesis* **14** 1493–1497.

Reddy BS, Hirose Y, Lubet R, Steele V, Kelloff G, Paulson S, Seibert K & Rao CV 2000*a* Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered

during different stages of carcinogenesis. *Cancer Research* **60** 293–297.

Reddy ST, Wadleigh DJ & Herschman HR 2000*b* Transcriptional regulation of the cyclooxygenase-2 gene in activated mast cells. *Journal of Biological Chemistry* **275** 3107–3113.

Reeves MJ, Newcomb PA, Trentham-Dietz A, Storer BE & Remington PL 1996 Nonsteroidal anti-inflammatory drug use and protection against colorectal cancer in women. *Cancer Epidemiology Biomarkers and Prevention* **5** 955–960.

Rigas B & Shiff SJ 2000 Is inhibition of cyclooxygenase required for the chemopreventive effect of NSAIDs in colon cancer? A model reconciling the current contradiction. *Medical Hypotheses* **54** 210–215.

Ristimaki A, Garfinkel S, Wessendorf J, Maciag T & Hla T 1994 Induction of cyclooxygenase-2 by interleukin-1 alpha. Evidence for post-transcriptional regulation. *Journal of Biological Chemistry* **269** 11769–11775.

Ristimaki A, Honkanen N, Jankala H, Sipponen P & Harkonen M 1997 Expression of cyclooxygenase-2 in human gastric carcinoma. *Cancer Research* **57** 1276–1280.

Robertson FM, Parrett ML, Joarder FS, Ross M, Abou-Issa HM, Alshafie G & Harris RE 1998 Ibuprofen-induced inhibition of cyclooxygenase isoform gene expression and regression of rat mammary carcinomas. *Cancer Letters* **122** 165–175.

Rolland PH, Martin PM, Jacquemier J, Rolland AM & Toga M 1980 Prostaglandin in human breast cancer: evidence suggesting that an elevated prostaglandin production is a marker of high metastatic potential for neoplastic cells. *Journal of the National Cancer Institute* **64** 1061–1070.

Rose DP & Connolly JM 1999 Omega-3 fatty acids as cancer chemopreventive agents. *Pharmacology and Therapeutics* **83** 217–244.

Sano H, Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai K, Kimura S, Kato H, Kondo M & Hla T 1995 Expression of cyclooxygenase-1 and -2 in human colorectal cancer. *Cancer Research* **55** 3785–3789.

Sasai H, Masaki M & Wakitani K 2000 Suppression of polypogenesis in a new mouse strain with a truncated $Apc^{\Delta 474}$ by a novel COX-2 inhibitor, JTE-522. *Carcinogenesis* **21** 953–958.

Sawaoka H, Tsuji S, Tsujii M, Gunawan ES, Sasaki Y, Kawano S & Hori M 1999 Cyclooxygenase inhibitors suppress angiogenesis and reduce tumor growth *in vivo*. *Laboratory Investigation* **79** 1469–1477.

Schreinemachers DM & Everson RB 1994 Aspirin use and lung, colon, and breast cancer incidence in a prospective study. *Epidemiology* **5** 138–146.

Schrey MP & Patel KV 1995 Prostaglandin E_2 production and metabolism in human breast cancer cells and breast fibroblasts. Regulation by inflammatory mediators. *British Journal of Cancer* **72** 1412–1419.

Shao J, Sheng H, Inoue H, Morrow JD & DuBois RN 2000 Regulation of constitutive cyclooxygenase-2 expression in colon carcinoma cells. *Journal of Biological Chemistry* **275** 33951–33956.

Sharpe CR, Collet JP, McNutt M, Belzile E, Boivin JF & Hanley JA 2000 Nested case-control study of the effects of non-steroidal anti-inflammatory drugs on breast cancer risk and stage. *British Journal of Cancer* **83** 112–120.

Sheng H, Shao J, Kirkland SC, Isakson P, Coffey RJ, Morrow J, Beauchamp RD & DuBois RN 1997 Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. *Journal of Clinical Investigation* **99** 2254–2259.

Sheng H, Shao J, Morrow JD, Beauchamp RD & DuBois RN 1998*a* Modulation of apoptosis and Bcl-2 expression by prostaglandin E_2 in human colon cancer cells. *Cancer Research* **58** 362–366.

Sheng H, Williams CS, Shao J, Liang P, DuBois RN & Beauchamp RD 1998*b* Induction of cyclooxygenase-2 by activated Ha-ras oncogene in Rat-1 fibroblasts and the role of mitogen-activated protein kinase pathway. *Journal of Biological Chemistry* **273** 22120–22127.

Simon LS, Weaver AL, Graham DY, Kivitz AJ, Lipsky PE, Hubbard RC, Isakson PC, Verburg KM, Yu SS, Zhao WW & Geis GS 1999 Anti-inflammatory and upper gastrointestinal effects of celecoxib in rheumatoid arthritis: a randomized controlled trial. *Journal of the American Medical Association* **282** 1921–1928.

Singh G 1998 Recent considerations in nonsteroidal anti-inflammatory drug gastropathy. *American Journal of Medicine* **105** 31S–38S.

Singh J, Hamid R & Reddy BS 1997 Dietary fat and colon cancer: modulation of cyclooxygenase-2 by types and amount of dietary fat during the postinitiation stage of colon carcinogenesis. *Cancer Research* **57** 3465–3470.

Soslow RA, Dannenberg AJ, Rush D, Woerner BM, Khan KN, Masferrer J & Koki AT 2000 COX-2 is expressed in human pulmonary, colonic, and mammary tumors. *Cancer* **89** 2637–2645.

Steinbach G, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su LK & Levin B 2000 The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *New England Journal of Medicine* **342** 1946–1952.

Stolina M, Sharma S, Zhu L & Dubinett SM 2000 Lung cancer cyclooxygenase-2 dependent inhibition of dendritic cells maturation and function. *Proceedings of the American Association of Cancer Research* **41** 619.

Subbaramaiah K, Telang N, Ramonetti JT, Araki R, DeVito B, Weksler BB & Dannenberg AJ 1996 Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells. *Cancer Research* **56** 4424–4429.

Subbaramaiah K, Chung WJ & Dannenberg AJ 1998*a* Ceramide regulates the transcription of cyclooxygenase-2. Evidence for involvement of extracellular signal-regulated kinase/c-Jun N-terminal kinase and p38 mitogen-activated protein kinase pathways. *Journal of Biological Chemistry* **273** 32943–32949.

Subbaramaiah K, Chung WJ, Michaluart P, Telang N, Tanabe T, Inoue H, Jang M, Pezzuto JM & Dannenberg AJ 1998*b* Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. *Journal of Biological Chemistry* **273** 21875–21882.

Subbaramaiah K, Altorki N, Chung WJ, Mestre JR, Sampat A & Dannenberg AJ 1999*a* Inhibition of cyclooxygenase-2 gene expression by p53. *Journal of Biological Chemistry* **274** 10911–10915.

Subbaramaiah K, Norton L, Gerald W & Dannenberg AJ 1999*b* Increased expression of cyclooxygenase-2 in HER-2-over expressing human breast cancer cells. *NCI 7th SPORE Investigators' Workshop.*

Subbaramaiah K, Hart JC, Norton L & Dannenberg AJ 2000 Microtubule-interfering agents stimulate the transcription of cyclooxygenase-2. Evidence for involvement of ERK1/2 and p38 mitogen-activated protein kinase pathways. *Journal of Biological Chemistry* **275** 14838–14845.

Suh N, Honda T, Finlay HJ, Barchowsky A, Williams C, Benoit NE, Xie QW, Nathan C, Gribble GW & Sporn MB 1998 Novel triterpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. *Cancer Research* **58** 717–723.

Suh O, Mettlin C & Petrelli NJ 1993 Aspirin use, cancer, and polyps of the large bowel. *Cancer* **72** 1171–1177.

Suzui N, Sugie S, Rahman KM, Ohnishi M, Yoshimi N, Wakabayashi K & Mori H 1997 Inhibitory effects of diallyl disulfide or aspirin on 2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine-induced mammary carcinogenesis in rats. *Japanese Journal of Cancer Research* **88** 705–711.

Takahashi Y, Kawahara F, Noguchi M, Miwa K, Sato H, Seiki M, Inoue H, Tanabe T & Yoshimoto T 1999 Activation of matrix metalloproteinase-2 in human breast cancer cells overexpressing cyclooxygenase-1 or -2. *FEBS Letters* **460** 145–148.

Tan WC, Privett OS & Goldyne ME 1974 Studies of prostaglandins in rat mammary tumors induced by 7,12-dimethyl-benz(a)anthracene. *Cancer Research* **34** 3229–3231.

Tardieu D, Jaeg JP, Deloly A, Corpet DE, Cadet J & Petit CR 2000 The COX-2 inhibitor nimesulide suppresses superoxide and 8-hydroxy-deoxyguanosine formation, and stimulates apoptosis in mucosa during early colonic inflammation in rats. *Carcinogenesis* **21** 973–976.

Thun MJ, Namboodiri MM & Heath CW Jr 1991 Aspirin use and reduced risk of fatal colon cancer. *New England Journal of Medicine* **325** 1593–1596.

Tiano H, Chulada P, Spalding J, Lee C, Loftin C, Mahler J, Morham S & Langenbach R 1997 Effects of cyclooxygenase deficiency on inflammation and papilloma development in mouse skin. *Proceedings of the American Association of Cancer Research* **38** 1727.

Tsujii M & DuBois RN 1995 Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell* **83** 493–501.

Tsujii M, Kawano S & DuBois RN 1997 Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *PNAS* **94** 3336–3340.

Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M & DuBois RN 1998 Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* **93** 705–716.

Tucker ON, Dannenberg AJ, Yang EK, Zhang F, Teng L, Daly JM, Soslow RA, Masferrer JL, Woerner BM, Koki AT & Fahey TJ 3rd 1999 Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer. *Cancer Research* **59** 987–990.

Vadlamudi R, Mandal M, Adam L, Steinbach G, Mendelsohn J & Kumar R 1999 Regulation of cyclooxygenase-2 pathway by HER2 receptor. *Oncogene* **18** 305–314.

Wadleigh DJ, Reddy ST, Kopp E, Ghosh S & Herschman HR 2000 Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. *Journal of Biological Chemistry* **275** 6259–6266.

Wiese FW, Thompson PA & Kadlubar FF 2001 Carcinogen substrate specificity of human COX-1 and COX-2. *Carcinogenesis* **21** 5–10.

Williams CS, Luongo C, Radhika A, Zhang T, Lamps LW, Nanney LB, Beauchamp RD & DuBois RN 1996 Elevated cyclooxygenase-2 levels in Min mouse adenomas. *Gastroenterology* **111** 1134–1140.

Williams CS, Tsujii M, Reese J, Dey SK & DuBois RN 2000*a* Host cyclooxygenase-2 modulates carcinoma growth. *Journal of Clinical Investigation* **105** 1589–1594.

Williams CS, Watson AJ, Sheng H, Helou R, Shao J & DuBois RN 2000*b* Celecoxib prevents tumor growth *in vivo* without toxicity to normal gut: lack of correlation between *in vitro* and *in vivo* models. *Cancer Research* **60** 6045–6051.

Wilson KT, Fu S, Ramanujam KS & Meltzer SJ 1998 Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and associated adenocarcinomas. *Cancer Research* **58** 2929–2934.

Wolff H, Saukkonen K, Anttila S, Karjalainen A, Vainio H & Ristimaki A 1998 Expression of cyclooxygenase-2 in human lung carcinoma. *Cancer Research* **58** 4997–5001.

Xie W & Herschman HR 1995 v-src induces prostaglandin synthase 2 gene expression by activation of the c-Jun N-terminal kinase and the c-Jun transcription factor. *Journal of Biological Chemistry* **270** 27622–27628.

Xie W & Herschman HR 1996 Transcriptional regulation of prostaglandin synthase 2 gene expression by platelet-derived growth factor and serum. *Journal of Biological Chemistry* **271** 31742–31748.

Yamada M, Kawai M, Kawai Y & Mashima Y 1999 The effect of selective cyclooxygenase-2 inhibitor on corneal angiogenesis in the rat. *Current Eye Research* **19** 300–304.

Yamamoto K, Arakawa T, Ueda N & Yamamoto S 1995 Transcriptional roles of nuclear factor κB and nuclear factor-interleukin-6 in the tumor necrosis factor α-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. *Journal of Biological Chemistry* **270** 31315–31320.

Yamamoto Y, Yin MJ, Lin KM & Gaynor RB 1999 Sulindac inhibits activation of the NF-κB pathway. *Journal of Biological Chemistry* **274** 27307–27314.

Yan Z, Subbaramaiah K, Camilli T, Zhang F, Tanabe T, McCaffrey TA, Dannenberg AJ & Weksler BB 2000 Benzo[a]pyrene induces the transcription of cyclo-oxygenase-2 in vascular smooth muscle cells. Evidence for the involvement of extracellular signal-regulated kinase and NF-kappaB. *Journal of Biological Chemistry* **275** 4949–4955.

Yin MJ, Yamamoto Y & Gaynor RB 1998 The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature* **396** 77–80.

Yoshimura R, Sano H, Masuda C, Kawamura M, Tsubouchi Y, Chargui J, Yoshimura N, Hla T & Wada S 2000 Expression of cyclooxygenase-2 in prostate carcinoma. *Cancer* **89** 589–596.

Zhang F, Subbaramaiah K, Altorki N & Dannenberg AJ 1998 Dihydroxy bile acids activate the transcription of cyclo-oxygenase-2. *Journal of Biological Chemistry* **273** 2424–2428.

Zhang X, Morham SG, Langenbach R & Young DA 1999 Malignant transformation and antineoplastic actions of non-steroidal antiinflammatory drugs (NSAIDs) on cyclooxygenase-null embryo fibroblasts. *Journal of Experimental Medicine* **190** 451–459.

Zhang X, Morham SG, Langenbach R, Baggs RB & Young DA 2000*a* Lack of cyclooxygenase-2 inhibits growth of terato-carcinomas in mice. *Experimental Cell Research* **254** 232–240.

Zhang Z, Sheng H, Shao J, Beauchamp RD & DuBois RN 2000*b* Posttranscriptional regulation of cyclooxygenase-2 in rat intestinal epithelial cells. *Neoplasia* **2** 523–530.

Zhao Y, Agarwal VR, Mendelson CR & Simpson ER 1996 Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE_2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene. *Endocrinology* **137** 5739–5742.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 26 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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